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“Aqueous Ammonia Soaking as a Pretreatment of Lignocellulosic Biomasses for Improving Manure-based Anaerobic Digestion”

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PhD Thesis

July 2017

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

“Aqueous Ammonia Soaking as a Pretreatment of Lignocellulosic Biomasses for Improving Manure-based Anaerobic Digestion”

A dissertation

Presented to the Department of Chemical and Biochemical Engineering

of the Technical University of Denmark

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

Anna Lymperatou

July 2017

Preface

This PhD dissertation is submitted to the Department of Chemical and Biochemical Engineering of the Technical University of Denmark (DTU), in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work presented in this dissertation was realized within the period of 2013-2017 and was carried out at the Department of Chemical and Biochemical Engineering of DTU (June 2015 – June 2017, Center PILOT PLANT and Center for Bioprocess Engineering, BIOENG) and at the Department of Chemistry and Bioscience of Aalborg University Copenhagen (Section for Sustainable Biotechnology, October 2013 – May 2015). This work was part of the project "AMMONOX - Ammonia for Enhancing biogas Yield and Reducing NO_x", funded by Energinet.dk. (Project N^o 12069). This thesis was supervised by Associate Professor Ioannis Skiadas (PILOT PLANT) and co-supervised by Associate Professor Hariklia Gavala (BIOENG).

The dissertation is structured in nine chapters. The 1st chapter presents a general overview of the background of the research in this Thesis and the specific subjects of the following study are briefly introduced. The 2nd chapter states the scope of this thesis. Chapter 3-6 present the main findings and conclusions of the research performed during this Thesis. The 7th and 8th chapters present the overall conclusions of the Thesis and future perspectives respectively. Finally, in chapter 9 the scientific papers and manuscripts that were concluded from the research carried out during this Thesis are presented.

List of papers

I. A. Lymperatou, H.N. Gavala, K.H. Esbensen, I.V. Skiadas. "AMMONOX: Ammonia for Enhancing Biogas Yield & Reducing NO_x – Analysis of effects of Aqueous Ammonia Soaking on Manure Fibers", Waste and Biomass Valorization 6, 449-457

II. Lymperatou, A., Gavala, H.N. Esbensen, K.H., Skiadas, I.V. "Screening for the important variables of aqueous ammonia soaking as a pretreatment method for enhancing the methane production from swine manure fibers" Extended Abstract in Proceedings of 14th World Congress on Anaerobic Digestion, 2015.

III. A. Lymperatou, H.N. Gavala, I.V. Skiadas. "Optimization of Aqueous Ammonia Soaking of Manure Fibers by Response Surface Methodology for unlocking the methane potential of swine manure" (accepted for publication in "Bioresource Technology")

IV. A. Lymperatou, H.N. Gavala, I.V. Skiadas. "Optimization of Aqueous Ammonia Soaking at Ambient Temperature for Enhancing the Methane Yield of Wheat Straw" (submitted)

V. A. Lymperatou, H.N. Gavala, I.V. Skiadas. "Effect of optimized Aqueous Ammonia Soaking of manure fibers on continuous anaerobic digestion of manure" (in progress)

Anna Lymperatou

Kgs.Lyngby, 24th of July 2017

Acknowledgements

A PhD Thesis, while depicts the path followed and the personal development of the PhD candidate, is a result of many other people's work and support. Thinking about my journey for obtaining this PhD degree, this Holy Grail, I would like to thank everyone who has contributed to the realization of this study and who has helped me one way or another to reach this very moment.

First of all, I would like to thank my supervisors Assoc. Professor Ioannis V. Skiadas and Assoc. Professor Hariklia N. Gavala for giving me the opportunity to work on this PhD project, but also for their guidance and stimulating conversations during the last three years.

I would like to thank Esperanza Jurado, who has facilitated my first steps on the subject of my Thesis with her previous work, and who has given me valuable advice on how to approach the stressful process of a PhD study.

I would also like to thank Kim Esbensen for our collaboration during the first year of this PhD study. Thank you for the interesting conversations and suggestions regarding complex interactive systems and representative sampling issues, especially when working with "nasty" semiliquid substrates as the ones I had to deal with.

Next, I would like to thank the collaborators of the AMMONOX project, to which this PhD study was linked, for their valuable input on the process proposed from an industrial point of view, and the interesting discussions during our meetings throughout the last three years.

This PhD study was partly carried out at Aalborg University Copenhagen (Section for Sustainable Biotechnology) and partly at the Technical University of Denmark (PILOT center and BioEng center) giving me the opportunity to meet many nice people. I would like to thank all of you who have welcomed me and who have facilitated my work during these years. Thank you for enriching my everyday life.

Special thanks to the BioEng center that has hosted me during the last two years and especially to Michael for being always available when needed. Special thanks also to Demi for the nice conversations and his assistance with the elemental analysis, and to Jorge for helping me with his practical approaches with the digesters when needed.

My closest colleagues and friends, Stavros, Anna and Cristiano thank you for our good collaboration all these years sharing together different laboratories with all the stressful and happy moments, and for the nice time shared, out of the university.

I would also like to thank my family for their love and support. Special thanks to my father for all the inspiring conversations we’ve had and to my mother for always supporting me for following my dreams and seeking new challenges.

I deeply thank my friends for showing a great understanding and support on my occasional “disappearance” due to high workload. Your friendship always gives me strength and balances my life, even though most of you are far away.

Most of all, I would like to thank Antonio for his support, love and understanding. Thank you for reminding me the inspirations for which I started this journey during the most stressful and disappointing moments.

Summary

Liquid manure is one of the most important sources of environmental pollution, contributing significantly to the anthropogenic Greenhouse Gas (GHG) emissions. The anaerobic digestion process of liquid manure is a mature technology that allows capturing emissions in the form of biogas, while simultaneously improving the characteristics of manure for soil application. Nevertheless, the anaerobic digestion process in biogas plants treating solely swine manure, is usually economically non-feasible due to the low conversion rate of the solid lignocellulosic fraction (manure fibers), and due to the high water and ammonia content. The pretreatment of manure fibers, or of other lignocellulosic biomasses, with Aqueous Ammonia Soaking (AAS) coupled to an ammonia recovery step, could potentially overcome these limitations when added to manure. However, the efficiency of AAS on increasing the methane yield of lignocellulosic biomasses may vary significantly depending on the conditions of the pretreatment applied.

The main objective of this thesis was to evaluate the efficiency of AAS on improving the methane yield of swine manure fibers when applied under different conditions. The importance of different AAS parameters was tested and the most influencing factors identified were the NH_3 concentration of the reagent, the duration of AAS and the solid-to-liquid (S:L) ratio. Heating up to 50°C during AAS did not produce any significant effect on the methane yield of pretreated fibers, allowing thus for a less energy intensive pretreatment process at ambient temperature (20°C). The AAS of swine manure fibers, depending on the conditions applied resulted in a variation of the methane yield from 90 to 214 ml/g TS after 17 days of batch anaerobic digestion. The optimal conditions for maximizing the methane yield corresponded to 7% w/w NH_3, 4 days of AAS and 0.16 kg fibers/l reagent, resulting in average to a 244% increase of the methane yield after only 17 days of digestion. Nevertheless, a strong interaction effect among the NH_3 concentration and the duration of AAS on the resulting methane yield was found, providing some flexibility to the process configuration. Empirical models were constructed for predicting the methane yield of manure fibers as a function of the levels of the AAS parameters. Based on these models, 5-11% w/w NH_3 and 3.8-6 days of AAS duration can be applied in order to obtain 95% of the maximum increase of methane yield.

The influence of the same AAS parameters as for manure fibers were also investigated on the methane yield of wheat straw, as this is an abundant lignocellulosic residue that could be considered for boosting the methane production of manure-based anaerobic digestion. The AAS of wheat straw

at ambient temperature (20°C) resulted in a variation of the methane yield between 223 to 325 ml/g TS after 17 days of batch digestion, depending on the conditions of AAS applied. The NH_3 concentration was found to be the most influencing factor for the efficiency of the AAS pretreatment of wheat straw, and the optimal conditions corresponded to 18% w/w NH_3, 7 days of duration and 50 g/l reagent, resulting in a 43% increase of the short-term methane yield (after 17 days of digestion). Strong interactions were identified among the NH_3 concentration and the duration of AAS, permitting a higher flexibility on the process configuration for increasing the short-term methane yield of pretreated wheat straw, as compared to swine manure fibers. According to the results obtained, a 95% of the maximum increase of methane yield can be obtained by pretreating wheat straw with 7.3-29% w/w NH_3 concentration for 3.5-7 days.

In an attempt to better understand how the biomasses investigated in this Thesis were affected by AAS under optimal conditions, an evaluation of the compositional changes that occurred due to the pretreatment was carried out. The results obtained, showed that no lignin removal took place on swine manure fibers, in contrast to wheat straw where limited removal was observed (9%). The hemicellulose faction of both biomasses was significantly solubilized (37% for swine manure fibers and 62% for wheat straw), an effect that is hypothesized to promote a better access of enzymes to carbohydrates improving thus the hydrolysis rate of the biomasses and their conversion to methane.

The performance of the optimally AAS-treated manure fibers on continuous manure-based anaerobic digestion was evaluated, as compared to the digestion of manure enriched with untreated manure fibers. A significantly improved performance of the digester running on manure enriched with optimally AAS-treated fibers was observed in all aspects. Based on the experiments run, an 18% and 38% increase of the biogas productivity and methane yield respectively can be achieved by pretreating manure fibers under optimal conditions. Additionally, a higher reduction of all organic components (carbohydrates, lipids, proteins, and lignin) can be achieved as compared to untreated fibers, being cellulose the fraction most significantly affected (42% increased reduction efficiency).

Overall, the results obtained in this Thesis, contribute to a better understanding of the potential and flexibility of the AAS pretreatment for ensuring high methane yields of the lignocellulosic biomasses tested. A systematic experimental procedure was followed for evaluating the effects of different AAS parameters on the methane yield of manure fibers, including the exploration, screening and finally optimization of the most influencing AAS parameters for ensuring high methane yields through anaerobic digestion. Wheat straw was considered as an alternative biomass for improving manure-based anaerobic digestion, and the effects of the AAS parameters on the methane yield of wheat straw were also extensively studied. Empirical models were produced for facilitating a techno-economic analysis of the AAS process of swine manure fibers as well as of wheat straw and for providing valuable information on the process flexibility and limitations prior to scaling up. Additionally, the compositional analyses of the optimally pretreated biomasses contributed to a better understanding of the mechanism of AAS under optimal conditions. Finally, the continuous anaerobic digestion experiments demonstrated that a higher reduction efficiency of the organic compounds is possible when swine manure is enriched with optimally AAS-treated manure fibers as compared to untreated manure fibers.

Dansk Sammenfatning (Danish summary)

Flydende gødning er en af de vigtigste kilder til miljøforurening og bidrager væsentligt til menneskeskabte drivhusgasemissioner. Bioforgasningsprocessen af flydende gødning er en moden teknologi, der gør det muligt at fange emissioner i form af biogas, samtidig med at gødningens egenskaber forbedres med hensyn til anvendelse som jordforbedring. Ikke desto mindre er bioforgasningsprocessen i biogasanlæg, der kun behandler svinegylle, ikke økonomisk mulig på grund af den lave nedbrydningshastighed af den faste lignocellulosefraktion (gyllefibre) og på grund af gyllens høje vand og ammoniakindhold. Adskillelsen og forbehandling af gyllefibre med vandig ammoniakopblødning (AAS, Aqueous Ammonia Soaking) efterfulgt af ammoniakfjernelse er tidligere blevet foreslået for at overvinde disse begrænsninger. Forøgelse af metanudbyttet af gødningsfibre ved hjælp af AAS kan imidlertid variere betydeligt afhængigt af de anvendte betingelser.

Hovedformålet med denne afhandling er at evaluere hvor effektivt AAS anvendt under forskellige betingelser kan forbedre metanudbyttet af svin gyllefibre. Indflydelsen af forskellige AAA parametre blev undersøgt og de faktorer med størst effekt blev fundet til at være var ammoniakkoncentrationen, varigheden af AAS og forholdet mellem faststof og væske (*S:L*). Behandlingen af svin gyllefibre med AAS resulterede i en variation af metanudbyttet efter 17 dages fordøjelse fra 90 til 214 ml/g TS. De optimale betingelser for maksimering af metanudbyttet svarede til 7% (vægt/vægt) NH_3, 4 dages AAS og 0,16 kg/l reagens, hvilket resulterede i 244% forøgelse af metanudbytte i 17 dages fordøjelser. En stærk vekselvirkning mellem ammoniakkoncentration og varighed på det resulterende udbytte giver en vis fleksibilitet i processen. Tilsvarende syntes det højeste S:L-forhold for AAS (0.28 kg/l) at være næsten lige så effektivt som det optimale S:L-forhold. Eksperimenter med kontinuerlig bioforgasning af svingødning beriget med gyllefibre viste, at når fibrene er optimalt forbehandlet, kan en henholdsvis 18% og 38% øgning af biogasproduktion og metanudbytte opnås i 18 dages retentionstid. Fjernelsen af cellulose blev signifikant forbedret, når svin gyllefibre blev forbehandlet. Der blev opnået 41% bedre fjernelseseffektivitet i sammenligning med ubehandlede gyllefibre. Desuden blev det vist at fordøjeligheden af gyllefibre der tilsættes svingødning er vigtigere end kun at reducere den samlede ammoniakkoncentration.

Hvede halm blev betragtet som en alternativ lignocellulosic biomasse, der kunne fordøjes med gødning til forbedring af biogasproduktionen. AAS af hvedestrå ved omgivelsestemperatur (20°C)

varierede mellem 223 og 325 ml/g TS af metanudbytte efter 17 dage afhængigt af de forbehandlede betingelserNH3-koncentrationen viste sig at være en kritisk faktor for forbehandlingens effektivitet, og de optimale betingelser svarede til 18% (vægt/vægt) NH_3, 7 dages varighed og 50 g/l reagens, hvilket førte til en 43% forøgelse af kortfristet metanudbytte. De stærke interaktioner, der er påvist mellem NH_3-koncentrationen og AAS-varigheden, tillader stor fleksibilitet for de procesparametrene der kan forøge det kortfristede metanudbytte fra forbehandlet hvedestrå.

AAS-forbehandling resulterer sædvanligvis i fjernelse af lignin samt en delvis opløseliggørelse af kulhydrater i lignocellulose biomasser. Ændringerne i biomassens sammensætning under forbehandlingen blev evalueret for bedre at forstå virkningerne af forbehandlingen under optimale betingelser. De opnåede resultater viser, at hemicellulose fraktionen af begge biomasser blev signifikant solubiliseret. Derfor blev det hypotetiseret, at denne virkning fremmer en bedre adgang for enzymer til cellulose, der forbedrer hydrolysehastigheden af biomasse og deres omdannelse til metan.

Resultaterne opnået i denne afhandling viser betydningen af en grundig undersøgelse af effekten af AAS's operationelle parametre på forskellige biomasser for at sikre høj effektivitet ved at øge deres metanudbytte. Forbehandlingens effektivitet afhænger meget af den behandlede biomasse. Svin gyllefibre viste sig at være gode receptorer af den foreslåede teknologi, medens der blev fundet en højere fleksibilitet for hvedestrå. AAS koblet til en ammoniakfjernelsesteknologi blev bekræftet at fremvise et stort potentiale til forbedring af energigenvinding af lignocellulose biomasser i bioforgasningprocesser baseret på gylle.

Table of Contents

1. Introduction

1.1. Swine manure production, management and pollution

In the last decades, the meat production sector has increased significantly as a result of the shift of the modern world consumption patterns to livestock products [1,2]. The European Union (EU) is currently the second largest pig meat producer after China, reaching a total pig meat production of 23 million tons only in 2015 [3]. Trends show that small producing units are disappearing while more intensive pig farming is favored [4]. Consequently, certain regions are presented with high animal concentrations, facing a great challenge to reduce the resulting environmental burden of livestock manure (Fig.1) [5].

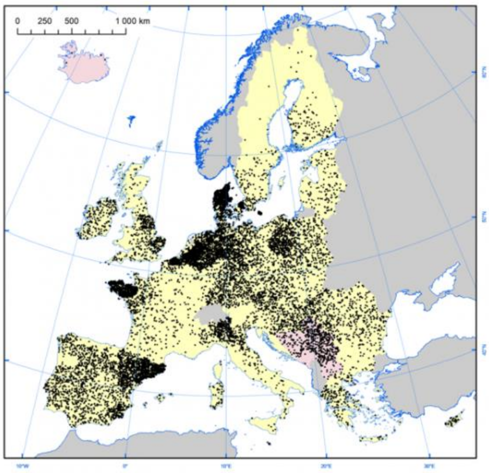

Figure 1 Sow density in the European Union of 2013 [4]

1.1.1. Swine manure as a source of soil fertilizing and environmental pollution

Livestock manure contains residual organic matter that is not degraded during the animal digestion and thus is not stabilized biologically. The further degradation of the organic matter occurs naturally, under both anaerobic and aerobic conditions and during the entire chain of manure management, i.e. from storage and processing until final disposal. The microorganisms that manure already contains, in the presence of the available nutrients and water, slowly degrade the organic matter, producing a series of degradation products, which are precursors of environmental hazards, affecting air, water and soil quality [6].

The air quality is affected by livestock manure management mainly through emissions of CH_4, N_2O and NH_3. Both CH_4 and N_2O are Greenhouse gases (GHG), contributing significantly to the accelerated Global Warming, producing multiple concerns in nowadays society. The CH_4 emissions in the EU are mainly attributed to the enteric fermentation of animals and to manure management [7]. Enteric fermentation is the main source of emissions in the ruminant sector (cattle, etc.) [8] in contrast to the pig sector where the largest share derives from processes related to manure management [9]. According to FAO [2], the global pig production sector is responsible for the emission of 668 million tons CO_2-equivalents, 27.4% of which originate from manure management (mainly in the form of CH_4 during storage) along with an additional 7.9% associated to N_2O emissions from application and direct deposition of manure to soils. On the other hand, manure contributes significantly to NH_3 emissions, which affect largely the atmospheric chemistry as NH_3 is a precursor of acidification [10,11]. Swine manure contains high concentrations of NH_3 and the further mineralization of organic N increases the losses during manure management. Since NH_3 volatilizes rapidly, most of the NH_3 emissions occur in pig houses and when manure is applied to soil [12]. Together, the emissions of N_2O and NH_3 from livestock manure correspond to 40% of the global anthropogenic emissions [13]. While emissions occur during the entire life chain of manure, other important impacts occur mainly when it is applied to soil.

Manure contains N, P, K and other macro and micro-nutrients that are essential to plants. The incorporation of livestock manure to arable land (croplands and grasslands) presents various benefits to the quality and fertility of soils [14], a fact that has been long ago acknowledged as manure has been traditionally applied to arable soils. While short term studies on soils receiving manure report contradictory results on plant uptake, long-term studies (20-120 years) have shown that manure application can actually reach the same crop productivities as fertilizers [15]. Additionally, both the physical properties as well as the biological activity of soils are affected by manure incorporation. According to FAO [14], soils that receive manure as an amendment present among other an improved water-holding capacity, a reduction of erosion risk, and an increase of soil organic matter, while an increased microbial activity and diversion has also been reported [16]. In Denmark, the use of manure for fertilizing crops has reduced the N consumption from mineral fertilizers by up to 50% over the last 25 years [17]. However, the nutrients of manure are prone to losses through leaching and run off, contaminating thus the underground and surface waterbodies. More specifically, nutrients such as NO_3^- and PO_4^{-3} are easily leached from soil, generating eutrophication phenomena and affecting negatively the fresh water quality. Other pollution forms that are caused by manure application to soil and need to be monitored include heavy metal accumulation, excessive salt concentration and increase of the population of pathogen microorganisms, affecting the quality of soil and jeopardizing the food and feed production. Finally, major concerns have arisen lately on the fate of antibiotics that are used in pig rearing, as their presence in manure that is spread to soils contributes to the building of microbial antibiotic resistance [5,18,19]. In conclusion, whether manure is a hazard to the environment or a valuable resource of nutrients promoting plant production and soil quality depends on a series of factors such as the storage conditions, the further processing of manure, as well as, on following good agricultural practices [20].

1.1.2. Swine manure collection and management in Denmark

Denmark has a long history in the pig production sector which is an important income source nowadays. According to the “Danish Agriculture and Food Council” [21] more than 90% of the pig meat produced is exported to over 140 countries. As a result, this very small country with a surface area of 42,931 km^2 and a total pig production reaching 31.3 million of pigs [21] faces great challenges in managing the massive amounts of the manure produced. The total manure production in Denmark (based on amounts collected from all farming animals) has been reported to reach ca. 35 million tons annually, out of which almost 50% is swine manure [22]. Manure management becomes especially problematic in the region of Jutland (Middle and Northern Jutland) where the largest share of pigs reside (Fig.2).

The different options on swine manure management are related to its physical state, which is dependent on the housing system and more specifically on the flooring type of pig farms. This can be either solid with bedding material for absorbing the urine and feces of pigs, or slatted (partially or fully) where the excretions of the animals are drained to a pit under them. In the first case, the resulting manure is handled in a solid form (farmyard manure) and is removed periodically by scrapping. In the second case, the reduced bedding material in combination with washing water, results into liquid manure that can be stored indoors or sent outdoors with the use of slurry channels [5]. Most pig housing systems in Europe are based on partially slatted floors [23] and 65% of the manure is handled in liquid form [24]. This facilitates the further manure management in farm as it can be pumped easily and the energy requirements are lower than for handling solid manure [13].

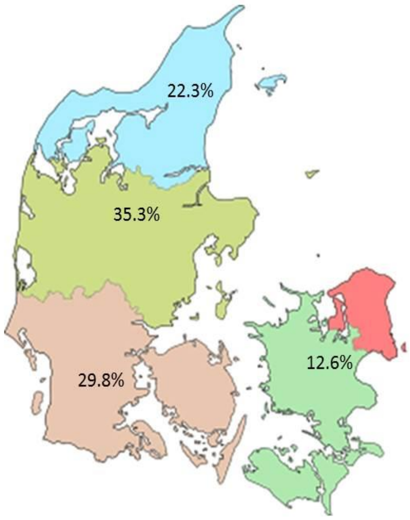

Figure 2 Distribution of pig population in Denmark 2015 [21]

In Denmark, similarly to the general European trends, the majority of swine manure is handled in liquid form (91% of the total manure production, [25]), and the regulations related to its management are rather strict. Pig farms are required to have extra facilities with sufficient capacity for storing manure for a minimum period of 6-9 months, as application to soil is prohibited from harvest to the 1st of February (with some exceptions) [26]. During storage, measures for avoiding gaseous N losses should take place such as the use of covers of the slurry tanks, the use of artificial or natural crust on the surface of tanks, and acidification [17]. Since 2006, farmers are obliged to report their annual fertilizing plan (Fertilizer Account), for which a large percentage of manure N has to be accounted for, restricting thus the use of additional fertilizers. Based on these regulations, the farmers should state the available area they own for manure spreading and this should be in accordance with the so called "harmony rules". According to these rules, the limit on spreading pig manure to land should not exceed 1.4 LU/ha

(LU: Livestock Unit, 1 LU ≈ 100 kg N). However, in Denmark many areas are designated as N vulnerable and stricter rules apply when the potential of reducing nitrate leaching is limited [27].

As a result of the intensive farming and given the environmental restrictions in certain regions, farmers are often in need of finding solutions alternative to local soil application. The available options are to export the excess of manure to other regions where spreading area is available or to send it for further processing to other facilities, e.g. a biogas plant. At biogas plants, manure is digested anaerobically, and as a result the volume to be handled is reduced, GHG emissions are captured, biogas production compensates economic pressure on farmers, and the digestate still rich in N can be redistributed to areas according to the harmony rules. In both cases, the transportation costs for liquid manure are rather high, a fact that has led to the application of separation technologies [28–30]. The separation of manure to a solid and a liquid fraction, permits transporting only the solid fraction (manure fibers) that is rich in organic matter and nutrients, reducing thus significantly the transportation costs [31,32].

1.1.3. Manure-based biogas plants in Denmark

Denmark has a long history in manure-based biogas production. In the early 1970s, in light of the forthcoming shortage of fossil fuel energy, the first farm-scale biogas plants were constructed [33]. In the 1980s the concept of centralized biogas plants was launched, where farmers of close vicinity would supply the plant with manure, and an improved biogas production was possible due to economy of scale and due to the possibility of incorporating more advanced technology [33,34]. Centralized plants started to operate on mixtures of liquid manure (75%) and other organic waste (25%) like residues from the food industry that significantly enhanced the biogas production and improved the economic performance of the process [22,35]. Until 2009, numerous biogas plants had been constructed, both farm-scale and centralized, and around 5% of the manure produced was used for biogas production [36].

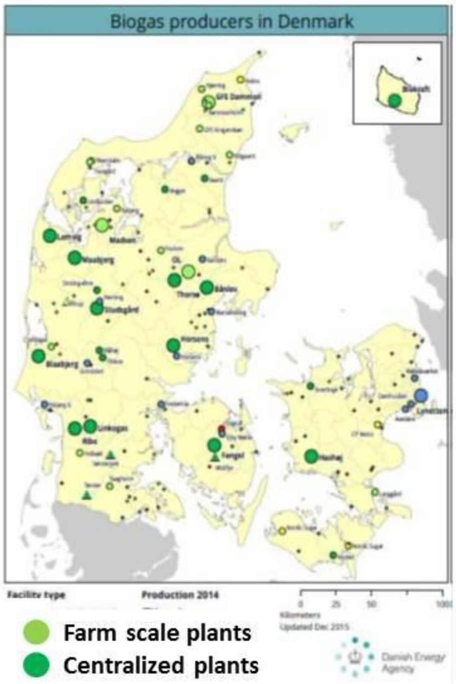

Figure 3 Manure-based biogas plants in Denmark 2015 [25]

In response to the EU regulations for reducing the environmental burden, and the need for expanding the share of renewable sources for energy production, the Danish government signed the “Agreement on Green Growth” in 2009 [37]. With this agreement an ambitious goal was set, to increase the amount of manure converted to energy up to 50% of manure produced until 2020, and ultimately up to 100%. This goal is expected to be met by the implementation of multiple new biogas plant projects and the expansion of currently existing plants, promoted by the higher economic support of the government through subsidies. In 2012, the amount of manure used for energy production did not exceed 6-7% of the manure produced [38,39], and an assessment of the new biogas plants led to the “Energy Agreement”, based on which further economic support for investments and more subsidies were provided [39]. Additionally, a Biogas Taskforce was formed for further encouraging the expansion of the biogas industry [30]. In 2014 ca. 8% of the manure produced was used for biogas production, originating mainly from 23 centralized and 48 farm-scale biogas plants (Fig.3) [25,40,41]. By the end of 2016, at least three more centralized biogas plants were constructed and more projects are on the way.

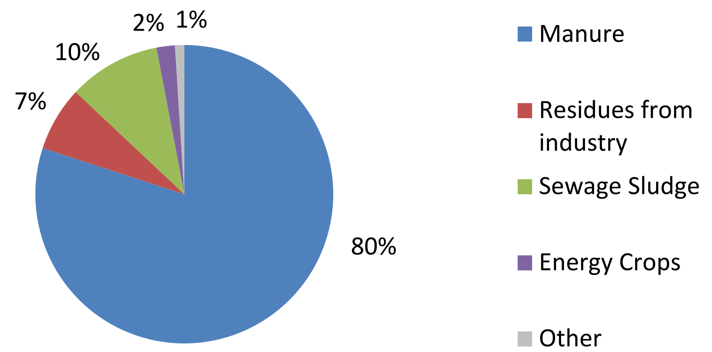

Figure 4 Contribution of feedstocks in biogas production in Denmark 2014 [25]

Based on estimations of the Biogas Taskforce, the biogas produced until 2020 is expected to increase from 5 PJ in 2014 to at least 10 PJ and possibly up to 16 PJ as more biogas projects are expected [42]. Nevertheless, in order to fulfill the ultimate goal, a great uncertainty on the availability of feedstocks is recognized, as the organic wastes that are traditionally used for co-digestion of manure have become scarce and the economic viability of plants processing solely liquid manure is rather poor [32,43]. Figure 4 shows the typical feedstocks used for biogas production in Denmark and the contribution of each feedstock to the total biogas production in 2014. The use of energy crops for co-digestion is not significant in Denmark (Fig.4) and its expansion is not encouraged, as from 2018 biogas plants will be eligible for subsidies when their share does not exceed 12% of the input, in comparison to the current limitation of 25% [44]. New alternatives are explored and significant research is carried out nowadays both on improving manure mono-digestion as well as for identifying potential co-substrates. According to recent reports, the further increase of biogas production is suggested to take place with the enrichment of liquid manure with solid manure (or separated solid fraction), straw, grass, aquatic biomass, household waste and catch crops among others [22,45]. Based on calculations of the available amounts of manure, excess straw and grass, the total biogas potential of Denmark corresponds to 26-51 PJ/year in 2030 [46], clearly showing the need for research on easing the exploitation of these substrates for manure-based biogas production.

1.2. Anaerobic Digestion

1.2.1. Introduction to Anaerobic Digestion - A multi-purpose process

Whether anaerobic digestion has first been applied by humans for energy production or for waste treatment and stabilization of organic matter still remains an unanswered question. According to anecdotal sources, biogas was used for heating bath water in ancient Assyria in the 10^{th} century BC [47,48]. Nevertheless, anaerobic digestion of solid waste has been reported to be used in China since 2000-3000 years ago, and the benefits of using mature manure (wetted and left for 6 months) instead of raw manure for fertilizing plants has been well-known since the 13^{th} century [47]. The first well documented information though tells us that it was in the 17^{th} century when van Helmont first observed that flammable gas is produced during the degradation of organic matter [49]. Later in 1776, Volta linked the amount of gas produced to the amount of organic matter degraded [49]. The fact that this flammable gas was CH_4 was discovered around 1804 - 1808 by Dalton and Davy independently [50]. In the 1890s, the first applications for treating wastewater took place, when the term “septic tank” was introduced [50]. During the same period, the first anaerobic digesters for producing energy were constructed in India and New Zealand, while in Exeter the biogas produced from the sewage sludge was used for lighting the town [48]. Since then, anaerobic digestion applications have generally flourished for energy production in developing countries, while in Europe they have been used mainly for wastewater treatment [51]. However, in light of the necessity to rely on renewable energy resources in the near future, a sharp increase in anaerobic digestion plants aiming at producing biogas has been noted in Europe. In conclusion, this dual purpose of anaerobic digestion in combination to the versatile uses of the two main products, biogas and digestate, depicts the potential of this process that makes it relevant regardless times and circumstances.

Anaerobic digestion for biogas production

The anaerobic digestion of organic substrates is a complex biological process during which organic matter is degraded by multiple microorganisms in the absence of oxygen. During the process, the C content of the organic matter is converted to its most reduced form, CH_4, and to the most oxidized one, CO_2, which are the main components of biogas. Other gases that are present in minor quantities include water vapor, O_2, H_2S, NH_3 and H_2 [52]. Biogas can be used directly for cooking and heating, a practice that is common for small-scale digesters. In larger scale anaerobic digestion, the most usual practice is the conversion of biogas to heat and electricity through combined heat and power (CHP) installations [53]. The heat produced is partly utilized in situ for covering needs of the process itself, and the rest of it can be distributed through district heating networks to consumers during cold seasons [54]. Other uses of biogas include upgrading processes, where the CO_2 and water content are reduced and H_2S is removed for avoiding corrosion of the infrastructure. When the resulting biomethane is of high purity, it can be used similarly as natural gas and be injected in the natural gas grid. The latter is considered one of the easiest ways to incorporate biogas more extensively to the energy platform in Europe, as there would be no need for new infrastructure [24,55]. Another application is the use of upgraded biogas (biomethane) as a transportation fuel. Sweden is a pioneer in expanding this practice, with 26% of the biogas produced being used as a vehicle fuel [56]. All in all, the multiple possibilities of using biogas in combination with the high diversity of feedstocks that can be used, place this fuel in a central position of the future bioenergy sector [24].

Anaerobic digestion as a waste treatment

Apart from the energy recovery of organic matter, anaerobic digestion is also a waste treatment technology due to the improved characteristics of the digested material (digestate) that make its final disposal safer. In general, high quality digestates can be applied to soil similarly as composts. The main effects of anaerobic digestion on waste are reduction of volume, inactivation of pathogens, inactivation of weed seeds and reduction of odor among others [57,58]. Additionally, the organic matter is reduced and organically-bound nutrients are liberated [59]. Consequently, digestates present improved properties for soil application in comparison to raw feedstocks as the potential for GHG emissions is reduced [2] and nutrients such as N are present in forms that can be directly absorbed by plants. The quality of digestates is greatly related to the initial composition of the feedstocks and to the efficiency of the anaerobic digestion process. In Denmark, when digestates originate from manure-based anaerobic digestion with more than 75% manure in the input, the same regulations apply as for application of raw manure to soil [41]. A separation of the digestate into a solid and a liquid fraction is also common as it permits handling more efficiently the nutrient contents. The resulting liquid fraction is rich in N and K and can be used as a N-K fertilizer, while the fiber fraction can be used as a soil improver and P fertilizer [5,60]. The fiber fraction can also be further processed if necessary e.g. by composting [61,62]. Although soil application is the most common practice, a future expansion of the anaerobic digestion processes will probably lead to the exploitation of digestates for other applications within biorefinery concepts. For instance, the use of liquid digestate that is rich in nutrients can be used for algae cultivation or for nutrient recovery processes [63], while the fiber fraction can be further energetically exploited through combustion, incineration, further anaerobic digestion or fermentation to produce ethanol [64].

1.2.2. Process description

The anaerobic digestion process, also known as the biogas process, is carried out through a highly diverse group of microbes, and can generally be described in four main steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig.5). These steps are interdependent and all together define the final efficiency of the conversion of complex biomass to biogas. A brief description of each step is given below.

Hydrolysis

Feedstocks for anaerobic digestion may present a soluble and easily degradable fraction as well as complex particulate matter. While the former is easily available for further digestion, the particulate matter consisting of biopolymers and inert materials needs to be disintegrated and hydrolyzed into shorter chain compounds. Hydrolysis is carried out by extracellular enzymes of facultative or obligate anaerobic bacteria such as *Staphylococcus sp.*, *Clostridia sp.* and *Bacteroides sp.* [65]. These enzymes are either secreted to the bulk liquid [66] or attached to the cell [67] liberating substances that microbes can metabolize. The main biopolymers of complex particulate matter are carbohydrates, proteins and lipids.

The structural carbohydrates of biomass consist of cellulose and hemicellulose. The hydrolysis product of cellulose is glucose while the products of hemicellulose are more variable including xylose, arabinose, mannose, glucose, galactose and sugar acids. Cellulose and hemicellulose are linked to lignin, which is a complex aromatic polymer, recalcitrant to biodegradation, the presence of which greatly affects the hydrolysis of structural carbohydrates. Due to the complexity of the lignocellulosic structure, in anaerobic digestion processes with high lignocellulosic content, hydrolysis is considered to be the rate limiting step [68]. Apart from lignin, more factors affecting the extent of hydrolysis of lignocellulose have been identified and are further discussed in section 1.4.1. Proteins consist of polypeptides and are hydrolyzed by proteolytic bacteria that disrupt the peptide (or amide) bonds and release polypeptides and amino acids [69]. The extent of hydrolysis of proteins depends largely on their solubility and structure. More soluble proteins like globular proteins are considered easier to hydrolyze than fibrous proteins [66]. Finally, lipids are high molar mass polymers composed by fatty acids and glycerol. Lipids are hydrolyzed by lipases to long-chain fatty acids (LCFAs) and glycerol, and their hydrolysis rate depends mainly on their fatty acid chain length, and other factors such as surface tension and pH [70]. Generally, the hydrolysis of lipids has been reported to be faster in comparison to that of proteins and carbohydrates [71,72]. The glycerol released upon lipid hydrolysis is further degraded through acidogenesis, while LCFAs through acetogenesis.

Acidogenesis

The available monosaccharides, glycerol and amino acids are further converted to acids and alcohols through the acidogenesis step by fermentative bacteria. At this stage, the microbes involved are often the same bacteria that take action in the previous step of hydrolysis, and diversity is very high given the wide environmental conditions (temperature, pH) that bacteria can tolerate [65]. Monosaccharides are fermented through the Embden-Doudoroff-Parnas (EMP) pathway or through the Entner-Doudoroff (ED) pathway to pyruvate, and then through Acetyl CoA to Volatile Fatty Acids (VFAs) and alcohols [70]. Amino acid fermentation is carried out by proteolytic bacteria that often couple the reduction and oxidation of amino acids through the Stickland reaction [70]. During degradation of amino acids, the released carboxylic acids, NH_3 and CO_2 produce a natural buffer in the liquid. Generally, the fermentation products include VFAs like acetic acid, propionic acid and butyric acid, and H_2 and ethanol.

Acetogenesis

Acetogenesis is the step where production of acetic acid takes place, which is an important substrate for the final conversion of intermediate products to CH_4. During acetogenesis, VFAs and alcohols from the acidogenesis stage are converted into acetic acid and H_2 by acetogens (e.g. *Syntrophomonas wolfeii*). These reactions are endergonic and in order for the metabolism of the H_2-producing acetogens to be thermodynamically feasible, the partial pressure of H_2 should be kept low [73]. This is where a syntrophic relationship is built among H_2-producing acetogens and H_2-utilizing methanogens, also known as “interspecies H_2 transfer” [74]. Based on this, the metabolism of H_2-producing acetogens is thermodynamically possible (ΔG°<0) only when the H_2 partial pressure is regulated by H_2-consuming microbes. The lack of this regulation may result in accumulation of VFAs. Table 1 shows an example of the thermodynamic feasibility of reactions of H_2-producing and H_2-utilizing microbes carried out in syntrophic association. The low partial pressure of H_2 can be provided also from other H_2-utilizing microbes, like sulfate reducers or homoacetogens. The latter constitute another important source of acetate production as they reduce CO_2 by utilizing H_2 through the acetyl-CoA pathway [75]. Finally, LCFAs resulted from the hydrolysis of lipids are further degraded during acetogenesis through β-oxidation resulting in acetate and H_2. In contrast to other substrates, acetogenesis is the limiting step of LCFA digestion, due to their low water solubility [76].

Table 1 Thermodynamic feasibility of syntrophic acetate oxidation [77]

Reaction	ΔG°
$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 2H_2 + H^+$	+104.6 kJ/reaction
$4H_2 + H^+ + HCO_3^- \rightarrow CH_4 + 3H_2O$	-135.6 kJ/reaction
$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$	-31 kJ/reaction

Methanogenesis

Methanogenesis refers to the formation of CH_4 and is the last step of anaerobic digestion. Methanogens belong to the domain of archaea and are obligate anaerobic microbes and highly specialized as they use a small range of substrates for producing CH_4. The main substrates are acetate and H_2 plus CO_2 based on which two pathways result, the aceticlastic (eq.1) and the hydrogenotrophic (eq.2) pathway respectively. Other substrates that are converted to CH_4 to less extent are formate, methanol, methylamines and dimethylsulfide [78,79].

$$CH_3COOH \rightarrow CH_4 + CO_2 \ (eq.1)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \ (eq.2)$$

Aceticlastic methanogens belong to the genera *Methanosaeta* and *Methanosarcina* [80,81] and this pathway is responsible for more than 2/3 of the CH_4 produced biologically [80,82,83]. The hydrogenotrophic methanogens are more diverse with *Methanomicrobium*, *Methanococcus*, *Methanobacterium* and *Methanosarcina* among the most common identified genera [78,80,81]. Aceticlastic methanogens are more sensitive to environmental conditions, and in case of inhibition, acetate can also be converted to CH_4 by hydrogenotrophic methanogens in association to syntrophic acetate oxidizers (SAO). SAO bacteria convert acetate into H_2 and CO_2, and hydrogenotrophic methanogens further convert H_2 and CO_2 into CH_4. The growth rate of methanogens is considerably lower than the growth rate of microbes involved in the rest of the anaerobic digestion steps. Therefore, when easily degradable substrates are digested, methanogenesis is the rate-limiting step [84].

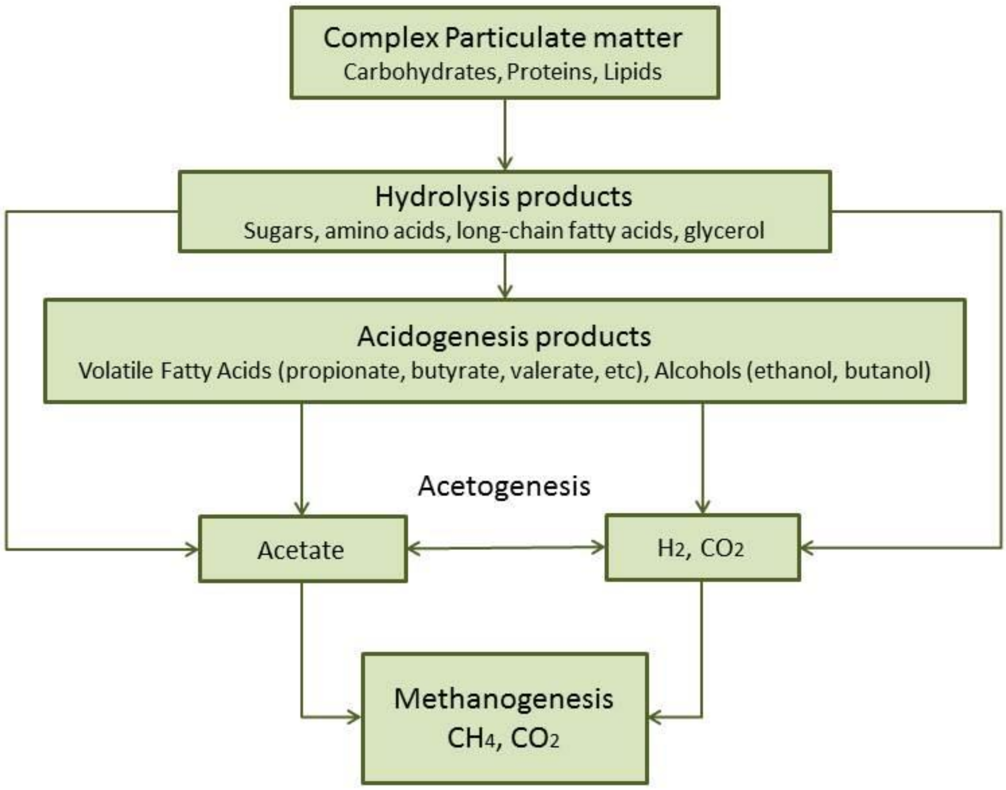

Figure 5 Overview of the Anaerobic Digestion steps of complex particulate matter (adapted from [85])

1.2.3. Process stability and configuration

As viewed in the previous section, anaerobic digestion is a complex process involving a large diversity of microbes. Methanogenesis is a process that takes place in nature in various places where anaerobic conditions exist, e.g. rice paddy fields, lake sediments, water-saturated soil, rumen, under glaciers, hot springs, etc. [86]. The temperature under which it can occur varies and can be roughly divided to psychrophilic (<20°C), mesophilic (20-45°C), thermophilic (45-60°C) and finally extreme thermophilic or hyper-thermophilic conditions (>60°C). Most processes in biogas plants operate under mesophilic or thermophilic conditions, both due to the higher diversity of microbes as well as for maintaining a high conversion rate taking into consideration the energy requirements. As in all biological systems, pH is a critical factor, defining whether microbes are active and performing their best or if they are inhibited. The different microbial groups involved in anaerobic digestion have different pH optima, and generally the range for a successful biogas production is around 6.5-8.5. However, methanogens are more sensitive to pH changes and the majority of them operate best in neutral environments (pH 7.0), with some exceptions [70,87]. Hydrolytic and fermentative microbes though, present a wider pH range and their activity is usually improved at pH values lower than 6 [67]. This difference has given rise to the so called two-step anaerobic digestion concept [88], where the hydrolysis and fermentation take place in one reactor, and the fermented material is sent to a second reactor for methanogenesis. This configuration permits adjusting operation to the optimal conditions for microbial growth of each step, improving thus the final conversion efficiency and preventing inhibition phenomena. Different configurations have also been introduced in order to overcome the long retention time of the material to digest. Usually, the retention time of the material digested corresponds to 12-25 days. Due to the low growth rate of methanogens, applying a lower hydraulic retention time (HRT) could lead to washout of cells and consequently to a failure of biogas production. A faster process can be achieved if the retention of microbes in the vessel is uncoupled from the retention of the material to digest. In such processes, the microbes are recirculated or retained in the digester tanks to allow for further growth.

The anaerobic digestion process is spontaneous under certain conditions, but can be inhibited in various ways leading to reduced conversion efficiency or to a complete failure of the process. Many toxic effects have been reported in the literature from a variety of compounds such as ammonia, sulfide, LCFAs, salts, lignin derivatives, heavy metals, light ion metals, chlorophenols, etc. [89,90]. The majority of substances inhibit the methanogens as this group is the most sensitive to environmental conditions. Inhibition of methanogenesis generally results in accumulation of VFAs, which causes a drop of pH inhibiting further the anaerobic digestion process. Consequently VFA concentration is considered a good indicator of stability and is used for monitoring anaerobic digestion processes together with pH. The risk of inhibition by these compounds depends on the origin of the feedstock and can also be greatly affected by the process parameters.

Ammonia is probably the most widely encountered inhibitor in anaerobic digestion processes, as N constitutes a basic element of organic matter. Nitrogen is an essential element for microbial growth. However, inhibition of methanogenesis may occur when feedstocks with high NH_3 concentration or high organic N content are digested (translated to high NH_3 concentration upon degradation). Total Ammonia Nitrogen (TAN) exists in equilibrium of free NH_3 and NH_4^+. Both free NH_3 and NH_4^+ have been identified as more inhibitory in various studies in the literature, though the majority of them report that free NH_3 is the main inhibitory form [91–93]. The concentration of free NH_3 depends on the TAN concentration, and increases with increasing temperature and pH. Thresholds for indicating ammonia inhibition vary greatly in the literature as such a phenomenon depends on many factors, among which are the source and acclimation of the inoculum. Nevertheless, a general limit of 3 g/l (TAN) has been reported for severe inhibition to occur regardless the conditions (pH, temperature) [94]. The assumption that NH_3 is the main inhibitor has been explained by alteration of the intracellular pH, either due to the ability of NH_3 to diffuse into the cell membrane producing instability of protons and deficiency of K^+, or due to inhibition of specific enzyme reaction [82,89,95,96]. The permeability of free NH_3 has also been used to explain the fact that round-shaped aceticlastic methanogens (e.g. *Methanosarcina*) have been found to be more tolerant to NH_3 than rod-shaped aceticlastic methanogens (e.g. *Methanothrix*), as the volume to surface area is greater resulting thus in less energy requirement for removing NH_3 from the cell [97]. Another contradiction in the literature regarding NH_3 inhibition in earlier studies is whether aceticlastic or hydrogenotrophic methanogens are more sensitive [89]. Nevertheless, the majority of recent works suggest that the aceticlastic pathway is the most affected [98–101].

1.3. Swine manure as a feedstock for anaerobic digestion

1.3.1. Characteristics and limitations of swine manure

The composition of liquid swine manure varies significantly among regions and countries as it depends on a series of factors, such as the feed and age of animals, the amount of bedding material used, the water added during management, and the duration and conditions of storage. Generally though, it presents a high water content (91-99%) [102] and the organic matter represents ca. 60-85% of the dry matter. The organic matter consists mainly of proteins, lipids and carbohydrates that are not degraded during animal digestion, along with soluble organic matter such as fatty acids, glycerol and ethanol produced by degradation of manure prior to processing. Table 2 shows the main characteristics of the swine manure used in the experiments of this Thesis. As commented in Section 1.1.1, degradation of the organic content occurs since the production of manure and until it is stabilized. Consequently, the fastest the manure is sent to biogas plants, higher is the CH_4 potential through anaerobic digestion, since less amount of organic matter is degraded during manure management. According to a recent assessment, up to 43% of the degradable organic matter of swine manure can be lost through emissions during storage in slurry pits [103].

Table 2 Composition of swine manure used in the experiments

Component	Swine manure used in experiments
Total Solids (% wet mass)	2.2
Volatile Solids (%wet mass)	1.5
Carbohydrates (% TS)	21.5
Proteins (% TS)	22.9
Lipids (% TS)	7.7
Lignin (% TS)	15.8
TAN (% TS)	1.09
C/N_{org} ratio	9.4

The anaerobic digestion of swine manure is often considered economically non-profitable and is discouraged [104,105] mainly for three reasons. First of all, the degradation of the organic fraction is very slow due to the refractory nature of lignocellulose, which is the main component, resulting thus in low productivity and to the necessity of using long HRTs. Then, swine manure usually presents a high NH_3 content due to degradation of organic nitrogenous compounds such as proteins and urea. The total N content of swine manure is often in the range of 1.2-8.2 g N/l [102], reducing thus considerably the biogas production due to partial inhibition. Finally, the water content is very high, a fact that results into very low organic loading rates in anaerobic digestion processes and thus in low biogas productivity. On the other hand, manure presents some properties such as high buffer capacity and the inherent presence of degrading microbes that make it a good substrate for anaerobic digestion. Due to the necessity of treating manure for avoiding negative environmental impacts, various approaches have been proposed for improving the biogas production and thus the profitability of the process. Some approaches aim at resolving only one of the three limitations presented above, while others aim at addressing all of them simultaneously. A short description of these approaches is given in the sequel.

1.3.2. Different approaches for improving swine manure biogas production

Process Configurations

A first approach for overcoming the limitations of swine manure anaerobic digestion is the application of different reactor configurations. Typically, batch and more often continuously-fed stirred tank reactors (CSTR) are used for manure-based anaerobic digestion. In CSTRs, the material is pumped into the reactor and simultaneously the digested material is removed, at the same flowing rate as the feeding under continuous stirring. This permits the homogenization of the reactor content and the suspension of microbes ensuring that they have a better access to substrates. Apart from batch and CSTR processes, other configurations proposed include Upflow Anaerobic Sludge Blanket (UASB), Anaerobic Filters (AF), anaerobic baffled reactors (ABR), plug flow reactors (PFR), anaerobic membrane bioreactors (AnMBR), and anaerobic sequencing batch reactors (ASBR) [106–112]. In such configurations, the HRT can be significantly shorter (down to a few days) with the solids retention time being significantly longer, resulting in increased cells concentration and improving thus considerably the CH_4 productivity of the process. For instance in a recent study where swine manure was treated in an AnMBR with 13 days HRT, an 83% increase of the CH_4 yield as compared to a CSTR was reported [112].

Nevertheless, in practice, the majority of the reactors used for swine manure anaerobic digestion are CSTR-type, being practically the only configuration used in Denmark both in centralized biogas plants as well as in farm-scale plants [39]. While other configurations may present improved productivities and result in larger amounts of manure treated, the need for changing the infrastructure in existing plants in combination with the more specialized control makes this solution difficult to implement.

Ammonia control and removal

Based on the assumption that free NH_3 is responsible for NH_3 inhibition, reduced inhibition can be obtained by regulating factors like the pH and the temperature of the digester. For instance, studies have shown that mesophilic (37°C) over thermophilic (55°C) temperatures can alleviate partial inhibition of swine manure digestion by reducing the share of free NH_3 in the liquid and increasing significantly the CH_4 yield [113]. The third parameter affecting the concentration of free NH_3 is the TAN concentration. In order to regulate TAN, the addition of materials with ion exchange capacity like minerals [114,115] and natural zeolites has been studied. Zeolites are able to adsorb NH_4^+, reducing thus the NH_3 and NH_4^+ concentrations in the liquid [116], and consequently improving the CH_4 productivity [117–119]. In a similar approach, the dilution of the influent manure has been suggested and successfully applied in order to reduce TAN concentrations and overcome inhibition [114,120]. Nevertheless, this practice reduces significantly the biogas productivity due to the reduced organic loading rate.

A more direct approach for regulating NH_3 inhibition is the implementation of NH_3 removal technologies. These include NH_3 stripping, struvite precipitation, flash distillation, evaporation, reverse osmosis, electrodialysis, and biological methods (such as the ANNAMOX process and nitrification-denitrification) among others [121–128]. The majority of these technologies though have been tested on digestates rather than prior to anaerobic digestion, and even when applied to raw manure, information on the effect of a subsequent anaerobic digestion process is scarce in the literature. The effect of NH_3 removal for enhancing CH_4 or biogas productivity has been studied mainly after NH_3 stripping, and small/medium scale applications have also been implemented [129]. During NH_3 stripping, manure is heated and the removal of NH_3 to the gas phase is facilitated by a gas that passes through the liquid, which can be air, steam, CO_2 or biogas, [130]. The liberated NH_3 can then be trapped by the use of acids, and salts such as ammonia sulfate, ammonia nitrate, or struvite (magnesium ammonium phosphate, MAP) are formed that can be used as fertilizers in agricultural soils. An important factor for the efficiency of NH_3 removal is the pH of the liquid that needs to remain alkaline for favoring the form of NH_3 against NH_4^+. Usually, addition of an alkaline reagent such as NaOH or lime is used for this purpose. Contradictory results though exist from laboratory experiments on the effect of NH_3 stripping to the CH_4 productivity of NH_3-stripped swine manure [122,128]. Nevertheless, the improvement of poultry manure mono-digestion by removal of NH_3 through an N extraction process (NiX®) has been successfully applied in full-scale anaerobic digestion plants [131] (Xergi A/S, http://www.xergi.com/biogas-plant/nix.html).

Increase of dry matter content

The high water content of manure affects significantly the biogas productivity, and more concentrated manure would be expected to result in a more feasible process. Wet anaerobic digestion can be operated at total solids (TS) concentrations of up to ca. 12% [55,132]. Drying or evaporation of manure is one approach for succeeding this high solids concentration. Nevertheless, it is not considered a good choice due to the high energy input needed for evaporating high water-content manures [133]. Alternatively, the addition of separated solids (manure fibers) to raw manure has been proposed for increasing the CH_4 production per unit volume [32,134–136]. Several separation technologies are available nowadays and, as commented in Section 1.1.2, this practice is extended in some European countries [129,133,135,137]. A large scale implementation of this approach has taken place in the past at the "Limfjordens bioenergy" biogas plant (former Morsø Bioenergi, Morsø, Denmark), though the productivity was not sufficiently improved as the N content reached inhibitory levels [132]. Instead of enriching manure with raw manure fibers, the recirculation of digested manure fibers separated from the effluent of anaerobic digestion processes has also been suggested for increasing the solids concentration [64,138,139]. This concept is based on the relatively short HRT applied in biogas plants that limits the extent of degradation of the fiber fraction. Consequently, by recirculating part of the digested fibers, more time is given to the recalcitrant fiber fraction to be digested, while the total solids concentration of the influent can be increased up to ca. 12%, improving thus the productivity of the process.

Co-digestion with lignocellulosic biomasses

The practice of co-digestion has a high potential to significantly improve the feasibility of manure-based anaerobic digestion and may result in an indirect increase of the % of manure treated by making the anaerobic digestion process more profitable and thus facilitating the installation of new manure-based biogas plants. As commented in section 1.1.4, easily degradable materials like residues from the food industry, household waste and energy crops are typically added to liquid manure for boosting the biogas production. Nevertheless, due to the limited availability of the easily degradable residues and due to land-use competition between food/feed production and production of energy crops, major focus has been given on agricultural residues. Examples of these are wheat straw, barley straw, rice straw, corn stover, etc. The availability of agricultural residues is very high even though part of these residues are incorporated to soil for maintaining organic C levels. According to Scarlat et al., if the amount of residues necessary for sustainable agricultural practices are taken into account, the energy potential of the remaining crop residues in Europe reaches 1537 PJ [140]. Part of this bioenergy potential is expected to be met by biogas production through anaerobic digestion. These materials have a larger biogas potential than manure and a significantly higher C/N ratio, resulting thus in suitable substrates for co-digestion with manure as they increase the CH_4 production and decrease the TAN concentration. Special focus has been given to agricultural straws due to the increasing dedication of arable land to cereal production [46]. Nevertheless, certain bottlenecks exist for the utilization of straws for wet anaerobic digestion such as low degradability rate due to their lignocellulosic content. Additionally the formation of floating layer presents another bottleneck [141] that is addressed with impregnation of straw prior to anaerobic digestion. Nowadays, only few biogas plants are able to use agricultural straws [22]. However, an expansion of this practice can be expected in the near future, as intensive research is carried out in order to improve the digestibility of these feedstocks and facilitate their incorporation to anaerobic digestion processes.

Pretreatment of manure

The low CH_4 productivity of manure is a result of the lignocellulosic content that is not easily degradable. In general, not more than 30-50% of the organic fraction of manure is degraded in biogas plants [142]. Thus many researchers have aimed at overcoming this limitation through pretreatment processes that facilitate manure digestion. Examples of pretreatments tested on swine manure include mechanical, thermal, and thermochemical pretreatments among others [143–146]. In some cases, significant improvement of CH_4 productivity or yield has been reported. Nevertheless, given that lignocellulose resides in the manure fibers, a pretreatment of separated manure fibers would be more attractive from an economic point of view, due to the significantly reduced volume of the material to be processed [134].

In conclusion, given the limited availability of easily degradable co-substrates, focus lies on the improvement of digestibility of manure fibers and agricultural residues. As the lignocellulosic content of this material restricts their biogas production, the application of a pretreatment process seems necessary for improving manure-based anaerobic digestion processes. During the late years, many different pretreatments have been proposed and tested for improving the CH_4 yield of lignocellulosic substrates. Currently, their actual application in large scale is limited due to economic restrictions. However, there is a large research effort on identifying pretreatments that could be successfully and economically applied to different lignocellulosic biomasses, and given the high potential of these feedstocks to be used for bioenergy production, pretreatment technologies will probably be used more extensively in the future.

1.4. Pretreatments of lignocellulosic biomasses

1.4.1. Parameters affecting the hydrolysis of lignocellulosic biomasses

Lignocellulosic biomasses are composed by carbohydrates (typically representing 55-75% TS [147]), lignin and in lower amounts pectin, proteins, ash and extractives. Biodegradation of lignocellulose occurs naturally, though as commented in section 1.2.2, its complex structure (Fig.6) impedes fast conversion, resulting thus in limited relevance for industrial applications [148]. Cellulose, the most abundant polymer in nature, consists of D-glucose units linked with β-1,4-glucosidic bonds and presents a high degree of polymerization [149]. The linear cellulosic chains are inter- and intra-connected with hydrogen bonds, forming microfibrils that result in closely packed and water insoluble elementary cellulosic fibers [147]. Cellulose contains parts that are highly crystalline and parts that are less structured (amorphous), the latter being considered easier to hydrolyze as high crystallinity is considered one of the limiting factors of cellulose hydrolysis [150,151]. In contrast to cellulose, hemicellulose is a branched heteropolymer consisting of C6 sugars (e.g. D-glucose, D-mannose, D-galactose), C5 sugars (e.g. D-xylose, D-arabinose) and sugar acids (e.g. glucuronic acid) [152]. Hemicellulose is linked to cellulose and lignin and is considered to be the "glue" of lignocellulosic biomasses providing them with extra rigidity [153]. Generally hemicellulose is characterized by a shorter degree of polymerization than cellulose [148] and is considered easier to hydrolyze [131]. Finally, lignin is a complex non-carbohydrate polymer consisting of phenylpropane units (p-coumaryl, coniferyl and sinapyl alcohol). It is the most recalcitrant component and serves as a natural barrier of plant biomass to microbial degradation. Under anaerobic conditions lignin can be degraded to some extent [154,155], with high molar mass lignin being more recalcitrant [156]. However this is a very slow process and thus lignin is often regarded as non-digestible. The covalent bonding of lignin-carbohydrate complexes presents an important factor that limits microbial attack [131]. Modelling of the CH_4 yield of lignocellulosic substrates as a function of their composition further supports the strong negative correlation of lignin to CH_4 generation [157]. Overall, the limited hydrolysis rate of lignocellulose is a result of the restricted access of enzymes to carbohydrates. While the importance of different factors influencing the access to carbohydrates seems to be related to the specific biomass in question, these could be generally summarized in the lignin content and distribution, the particle size, the moisture content, the degree of polymerization, the hydrophobicity and crystallinity of cellulose and the sheltering of cellulose by hemicellulose [131,147,148,152,158].

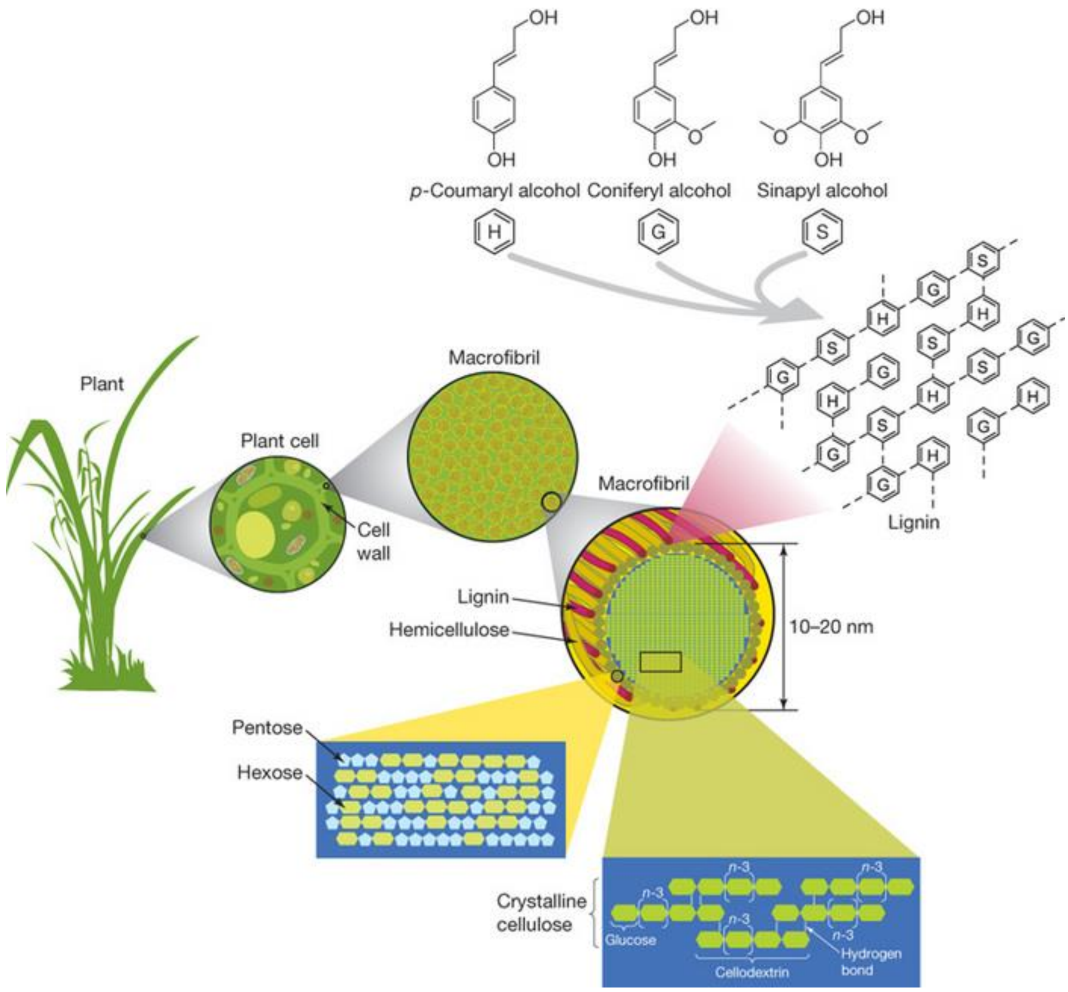

Figure 6 The structure of lignocellulose [159]

1.4.2. Different pretreatments

The different pretreatment methods suggested for facilitating hydrolysis of lignocellulose can be generally categorized into thermal, mechanical, chemical (acidic or alkaline) and biological methods. Combinations of these are also often examined (e.g. thermochemical, mechanical-chemical, biological-chemical) aiming at maximizing efficiency due to synergistic effects. The mode of action of pretreatments generally varies, though all of them aim at improving the access of enzymes to carbohydrates. In general, mechanical pretreatments e.g. grinding and milling, result in reduced particle size and degree of polymerization and in increased available specific surface area, facilitating thus enzymatic attack [160]. On the other hand, during thermal pretreatments, solubilization of hemicellulose initially occurs, which is the less thermally stable component, providing thus a better access to cellulose [152]. Chemical pretreatments involve acids or alkaline reagents, and depending on the conditions they may produce hemicellulose solubilization, lignin removal, and reduction of degree of polymerization and of cellulose crystallinity [161]. Finally, biological pretreatments such as enzymatic, bacterial and more often fungal pretreatments have shown to be very effective on producing degradation of lignin and occasionally of hemicellulose [162].

Table 3 Categories of different pretreatments and their general characteristics (based on [131,152,160,162]**)***

Pretreatment	Main effects	Key features	Examples
Thermal	Solubilization of hemicellulose Solubilization of lignin	❖ Possibility of using waste heat from gas engines ❖ Short duration ❖ High energy input ❖ Production of inhibitors	Simple thermal, steam explosion, microwave, Liquid hot water
Mechanical	Increase of available specific surface area, reduction of degree of polymerization	❖ Short duration ❖ Improved handling of feedstock ❖ High energy input	Milling, Grinding, maceration, extrusion
Acidic	Solubilization of hemicellulose, alteration of lignin structure	❖ High sugar release ❖ Necessity for chemicals recycling ❖ Corrosive ❖ Production of inhibitors	Sulfuric acid, nitric acid, hydrochloric acid
Alkaline	Lignin alteration or removal, partial hemicellulose solubilization, alteration and swelling of cellulose structure	❖ Limited loss of sugars ❖ Necessity for chemicals recycling ❖ Corrosive	Sodium hydroxide, ammonia, lime, potassium hydroxide
Biological	Lignin degradation, possible hemicellulose degradation	❖ Eco-friendly ❖ No risk of formation of inhibitory compounds ❖ Long duration ❖ Possible consumption of sugars	Fungal, bacterial or enzymatic (white-rot fungi, composting)

*General effects observed on different lignocellulosic biomasses and key features often associated to pretreatment categories

A pretreatment technology, apart from being efficient on improving the digestibility of a feedstock, is required to fulfill some additional characteristics. An undesirable effect is the excessive degradation of lignocellulose that leads to the formation of inhibitory by-products such as furfurals or 5-hydroxylmethulfurfurals (HMF) and phenolics [131]. Usually these inhibitors are formed during harsh conditions, such as concentrated acidic or high temperature pretreatments [152]. In the same line, avoiding the consumption of cellulose and hemicellulose (e.g. in biological treatments) and minimizing the production of waste streams are also of interest. Another possible requirement would be the efficient applicability of a certain pretreatment to different feedstocks [160]. For instance, this could be relevant in cases where biogas plants receive seasonal feedstocks (e.g. residues from seasonal crops) and would need to ensure flexibility of the process. Scalability of pretreatments is also important, as occasionally methods can be very efficient at bench scale but inconvenient to implement at industrial scale [148]. Finally, the economic feasibility is crucial for considering a pretreatment application. In this sense, low energy and water requirements are of interest as well as the limited use of chemicals [131]. Practically, all pretreatments present some advantages and limitations. In Table 3 a general overview of the main advantages and limitations of pretreatment categories is presented along with some examples of each kind.

Besides the variety of pretreatment methods employed in bench-scale, to date only few have been successfully applied at full scale, being these mainly thermal, mechanical and biological methods [131,163,164]. Chemical pretreatments are often very efficient on improving the digestibility of various feedstocks [161] by releasing sugars and, for example, this is the reason for which quantification of structural sugars of lignocellulosic biomasses is usually carried out after acid hydrolysis [151,165]. However, their implementation in the frame of an industrial biofuel production can be difficult to handle as chemicals may be toxic and corrosive, requiring special equipment and safety measurements. Additionally, the use of chemical reagents requires their recycling in order to be environmentally friendly and reduce the cost of the process; a requirement that is not always possible to fulfill. Among the various chemical reagents that have been tested for pretreating lignocellulosic biomasses, NH_3 is considered relatively easy to handle, less corrosive than e.g. sulfuric acid, and easy to be separated from the pretreated biomass due to its high volatility. These special characteristics of NH_3 pretreatments have been recognized by many researchers [166–170]. Moreover, there is a lot of experience in handling NH_3 in industrial applications (e.g. ammonia production plants and industrial refrigeration) that can prove valuable for facilitating the scaling up of the process. Finally, in comparison to other chemicals such as NaOH, the presence of NH_3 does not limit the quality of digested biomass (digestate) when this is applied to soils [131].

1.4.3. Ammonia Pretreatments

The first studies of NH_3 treatment of lignocellulosic biomasses took place more than 50 years ago, when agricultural straws were ammoniated initially for improving their quality as ruminant feed. The pretreated straws presented an increased N content and resulted in improved animal digestibility [171,172]. Since then, NH_3 pretreatments have also been tested for exploiting biomasses in the pulping industry [173] and for biofuel production [134,174–176]. More recently extensive research has been carried out mainly for ethanol production, giving rise to different configurations of NH_3 pretreatments, where the main varying parameters are the form of NH_3 (anhydrous or aqueous, liquid or gaseous), and the conditions applied (temperature and pressure).

Most NH_3 pretreatments involve high temperature and pressure conditions, being thus combinatorial methods. The Ammonia Freeze/Fiber Explosion (AFEX) process is one of the first configurations proposed for NH_3 treatment of lignocellulosic biomasses. During AFEX the biomass is treated with liquid anhydrous or highly concentrated aqueous NH_3 (> 70%) and high temperature and pressure are applied (50-120°C, ca. 15 atm) for a short period of time (5 min) [166,170,174,177]. In AFEX pretreatment, a sudden release of pressure at the end of the process produces expansion of the fibrous biomass, which generally improves the enzymatic access with minimum to null losses of biomass solids [170]. The Ammonia Recycle Percolation (ARP) process is based on the continuous recirculation of aqueous NH_3 flowing through a packed-bed (flow through type) reactor (percolation reactor) [178–180]. ARP involves application of both high temperature (150-210°C) and pressure (23 atm), conditions under which high lignin removal is obtained in combination with significant solubilization of hemicellulose [170]. The use of gaseous NH_3 has been tested in the concept of the Low-Moisture Anhydrous Ammonia (LMAA) pretreatment. During LMAA the biomass is initially left with anhydrous NH_3 at ambient conditions and subsequently the ammoniated biomass is heated to 40-150°C for 72-144 hours [170,181]. Another possibility is the supercritical NH_3 pretreatment (132.4°C, 111 atm), which has been suggested to improve the digestibility of biomass and constitutes one of the first configurations of NH_3 treatment tested [176]. Finally, probably the simplest NH_3 pretreatment tested so far for biofuel production is the Aqueous Ammonia Soaking (AAS or SAA) pretreatment [175,179]. During AAS, the biomass in question is soaked in an aqueous solution of NH_3 (1-32% w/w) in batch mode for the desired duration (few hours to days) at mild conditions (from ambient to 90°C, at atmospheric pressure). Subsequently the NH_3 reagent is removed either by evaporation (distillation or drying) or by washing the solids with water.

Extensive research has been carried out on different process configurations of NH_3 pretreatments for sugar release and enzymatic digestibility, though for biogas production only AAS has been tested on lignocellulosic biomasses [177]. AAS requires a low energy input as the conditions applied are milder than for other NH_3 pretreatments and is anticipated to be more economically viable. Additionally, while in some processes such as in bioethanol production the release of sugars is desirable in very short durations, in anaerobic digestion, which is a slower process milder pretreatment conditions may be equally efficient for increasing the digestibility of biomasses. So far, AAS has been tested in numerous biomasses for improving CH_4 generation and interest in this pretreatment appears to increase in the recent years. Table 4 presents an overview of the results obtained from AAS-pretreated biomasses destined for anaerobic digestion processes. The biogas or CH_4 yield increase of biomasses after AAS treatment ranges from insignificant to as much as 205%, the latter corresponding to application of AAS on digested manure fibers. Nevertheless, apart from different inocula used, there are two important factors complicating a fair comparison among the various studies, the first one being the NH_3 removal method followed. As AAS is necessarily followed by this step and the digestion tests are carried out after NH_3 removal, the method followed can highly influence the final yields. In this sense, many researchers have chosen to wash the pretreated biomass and digest only the solid fraction, while in other studies a distillation step is applied with subsequent digestion of both the solid and liquid fraction (Table 4). The second factor is the limited amount of studies where AAS was tested under different conditions (lack of optimization), limiting thus the evaluation of the potential of AAS on these biomasses.

Table 4 Effect of AAS on the CH_4 yield of different biomasses reported in literature

Biomass pretreated	Ammonia removal method	% Increase of CH_4 yield	References
Cattle manure fibers	Not reported	0-23	[134]
Switchgrass	Washing with water	65	[182]
Wheat straw	Different methods	11-56**	[183–185]
Rice straw	Not reported*	31-126	[186]
Swine manure fibers	Distillation	178	[167]
Digested manure fibers	Distillation	30-205	[139,167]
Miscanthus	Distillation	25-27	[184]
Willow	Distillation	94-162	[184]
Corn stover	Different methods	27-ca.78	[187,188]
Lawn grass	Washing with water and drying	20	[189]
Sunflower straw	Distillation	38	[168]
Poplar	Distillation	149	[168]
Grass	Distillation	26	[168]

*Direct use of biomass without further treatment is mentioned

**56% corresponds to increase of biogas yield

Mechanism of Ammonia action on lignocellulosic biomasses

Ammonia is considered to be highly effective on altering and removing the lignin fraction of biomasses. It has been suggested that NH_3 cleaves the ester bonds of lignin and the ether and ester bonds of lignin-carbohydrate complexes, providing thus an improved access of enzymes to cellulose [170,180,190–193]. A study performed by Gao et al. showed that the lignin fraction of wheat straw pretreated with NH_3 was significantly solubilized, though minimal structural changes were observed when the pretreated biomass was subjected to different analytical techniques (NMR, FTIR, Py-GC/MS) [191]. Another study on pretreated switchgrass showed that lignin removal and re-allocation occurred after AAS pretreatment [194]. Apart from lignin alteration or solubilization, NH_3 often results in the solubilization of hemicellulose to different extents, which has been hypothesized to increase the accessible pore volume and specific surface area [158] and facilitate access to cellulose [174]. On the other hand, the cellulose content is less affected and its retention after pretreatment can reach nearly 100% [195]. However, the partial removal of lignin and hemicellulose of liquid NH_3-treated biomasses can result into reorientation of the cellulose modifying thus its structure [158].

Liquid NH_3 appears to affect tremendously the crystallinity of cellulose by interaction of NH_3 molecules with the hydroxyl groups of the broken hydrogen bonds [196]. The change of crystallinity of cellulose (Fig.7) is achieved by increasing the volume of unit cells from 671 cubic Å to 801 cubic Å when a cellulose-ammonia complex is formed, and finally to 702 cubic Å after NH_3 removal [197]. The resulting allomorph of cellulose (cellulose III) has been suggested to be easier to hydrolyze than the initial cellulose (cellulose I_{β}) [147,193,198]. Figure 7 presents the mechanism of liquid NH_3 on cellulose as simulated by Bellesia et al. [199]. Additionally, there is evidence that the temperature plays a special role on the resulting crystallinity of NH_3-treated biomasses. Mittal et al. subjected cellulose samples of different crystallinity to NH_3 pretreatment and evidenced a highly crystalline structure under high temperature (up to 140°C), while the structure was rather amorphous at ambient temperatures [197]. In general it appears that the mechanism of NH_3 pretreatments on lignocellulosic biomasses is largely dependent on the conditions applied.

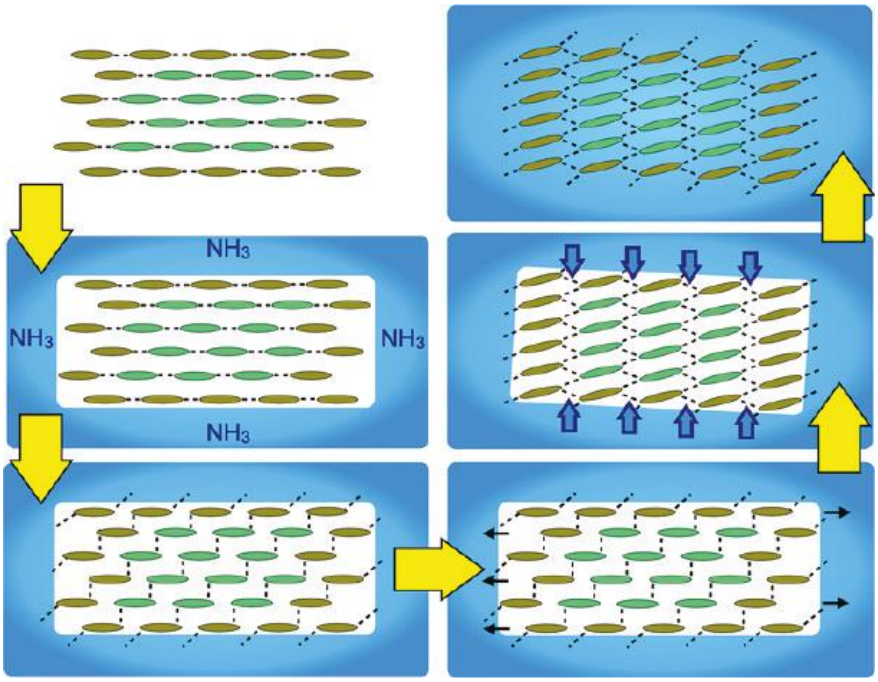

Figure 7 Mechanistic model presentation of the interaction of liquid NH_3 with cellulose fibrils. The initial redirection of the cellulose structure facilitates the penetration of NH_3 molecules to the cellulose structure, changing cellulose I_{β} to cellulose III [199].

AAS parameters and process considerations

The parameters of AAS can play a crucial role to the efficiency of the pretreatment on improving the biodegradation of a certain biomass, and the specific conditions and configuration can also be decisive to the economic viability of an industrial implementation. Thus, it is essential to evaluate the flexibility of the conditions of a pretreatment prior to considering its integration to anaerobic digestion processes.

The main parameters that can be adjusted in the AAS pretreatment are the temperature of the process, the concentration of NH_3 in the reagent, the duration of the pretreatment and the solid-to-liquid (S:L) ratio (mass of feedstock to volume of reagent). The temperature of NH_3 pretreatments largely affects the configuration of the process and increases the cost of an AAS implementation. Based on this, most studies of AAS application for bioconversion processes have tested the efficiency of the pretreatment up to 90°C. This level of temperature is considered to correspond to mild conditions and, in the context of biogas plants that are often equipped with CHP installations, residual heat could be used for fulfilling the energy requirements. Nevertheless, due to alternative uses of the waste heat for other applications such as hygienization of influent of the digester or distribution to neighboring houses, it could be desirable to avoid heat application. Furthermore, in a process that involves the use of NH_3 that needs to be removed, the waste heat could be used for facilitating an NH_3 recovery step. Regarding the effect of high temperature on the biomass, a strong correlation among heat application and lignin removal has been reported by different researchers on some lignocellulosic biomasses [185,200], and lignin reduction is a common observation on many NH_3-treated biomasses [185,201–203] with values reaching as high as 85% of reduction [179]. Given that this effect is considered to be one of the most efficient on improving digestibility, the effects and the efficiency of AAS at ambient or low temperature on different lignocellulosic biomasses present interesting topics, on which limited and contradictory information is currently available [168,188]. The NH_3 concentration is also expected to be a critical factor on the efficiency and economy of the pretreatment process. Interestingly, only few studies have tested different NH_3 concentrations on pretreated biomasses for anaerobic digestion. From a process point of view, this factor can largely affect the feasibility of the pretreatment, as high NH_3 concentrations are more difficult to handle (corrosive) and more costly if an efficient recycling of NH_3 is not possible. On the other hand, the duration of AAS may interact strongly with the

NH_3 concentration or the temperature of the pretreatment, and thus can be an interesting parameter for process configuration. For instance, a longer duration might permit reducing the severity of AAS, minimizing thus the energy and chemical input requirements, but increasing the space requirements and thus also the initial investment cost for the integration of the pretreatment to anaerobic digestion installations. Finally, the S:L ratio may affect both the contact of the NH_3-reagent with the biomass as well as the configuration of the process. A high S:L ratio is translated to an increased mass of feedstock pretreated per volume unit of reactor being thus preferable for industrial applications. In this sense, the initial investment cost of AAS of high durations could be compensated by high S:L ratios. In conclusion, given the nature of the AAS parameters, important interaction effects among them can be expected that could provide certain flexibility on the process design, making the assessment of these effects necessary.

2. Scope of this Thesis

As briefly introduced in Chapter 1, swine manure mono-digestion is usually an economically non-feasible process due to the low degradation rate of the solid fraction (manure fibers) and the high water and ammonia content, which result in a poor biogas production. Consequently, the largest fraction of manure produced nowadays is not digested anaerobically, producing a challenge to its management due to the resulting negative environmental consequences. The aqueous ammonia soaking (AAS) pretreatment of lignocellulosic biomasses to be digested with swine manure, when coupled to an ammonia removal step, could potentially provide a solution to these limitations. Previous studies have shown that AAS can significantly improve the methane yield of some lignocellulosic biomasses, though the conditions under which it is applied may influence significantly the efficiency of the pretreatment on different biomasses.

The main goal of this Thesis was to evaluate the potential and flexibility of the AAS process configuration on different lignocellulosic biomasses for increasing their methane yield. An efficient AAS pretreatment can lead to an increase of the amounts of manure treated anaerobically, either directly by enriching manure with pretreated raw or digested manure fibers, or indirectly by co-digestion of manure with pretreated agricultural straw, due to the improved manure-based biogas production. For this reason, the effects of different parameters of AAS (temperature, duration, ammonia concentration and solid-to-liquid ratio) on the methane yield of pretreated biomasses were systematically investigated in this study. It was hypothesized that the optimal conditions of AAS will differ among different biomasses and certain flexibility of the process configuration could be found based on interactions among the AAS parameters. Finally, an investigation of the continuous anaerobic digestion of AAS-treated manure fibers in comparison to untreated manure fibers, could indicate how AAS affects the reduction efficiency of the major organic components (e.g. carbohydrates, lipids and proteins).

The specific objectives of this Thesis were the following:

- To investigate the importance of the parameters of AAS that can potentially influence significantly the efficiency of AAS to increase the methane yield of digested manure fibers based on previously obtained results, in order to plan an optimization strategy.
- To identify which parameters of AAS are the most influencing on the resulting methane yield of pretreated raw swine manure fibers.

- To evaluate the effects of the most important parameters of AAS of swine manure fibers on the improvement of their digestibility and conclude on the optimal conditions leading to the maximum methane yield.
- To determine the optimal conditions of AAS of wheat straw at ambient temperature for maximizing the methane yield, and provide an insight on the flexibility of an efficient AAS pretreatment.
- To construct empirical models for the prediction of the methane yield of the pretreated biomasses (swine manure fibers and wheat straw) as a function of the parameters of AAS applied.
- To evaluate the effects of AAS on the composition of swine manure fibers and wheat straw when these are pretreated under optimal conditions.
- To test the performance of optimally-AAS treated swine manure fibers in continuous anaerobic digestion in comparison to non-treated manure fibers when these are enriched to swine manure, in regards to the biogas productivity and methane yield.
- To evaluate the effect of optimal AAS on the efficiency of reduction of the major organic components of pretreated swine manure fibers when added to swine manure in continuous anaerobic digestion.

In continuation a brief summary of the main results obtained from the studies carried out during this Thesis are presented, and the relevant papers can be found in Chapter 9.

3. Influence of AAS parameters on enhancing the Methane Yield of Digested Manure Fibers

3.1. Scope

The re-injection of digested manure fibers (separated from anaerobically digested manure) to the anaerobic digester can address both the low dry matter content of swine manure and increase the retention time of the substrate in the digester, improving thus partly the biogas productivity of the process. However, in order to improve the biodegradation rate of digested manure fibers, which is the most recalcitrant fraction of manure remaining after a first anaerobic digestion process, a pretreatment step is needed. As viewed in section 1.4.3, AAS has been applied to various lignocellulosic biomasses for improving their methane yield, and digested manure fibers are the biomass that best has responded to this pretreatment. Still, a large variability of the ultimate CH_4 yield from 30 to 205% has been reported after AAS under different conditions [139,167]. In this study, an exploration of previously obtained experimental results was carried out, in order to find correlations of the increase of the CH_4 yield with different parameters of AAS, in the frame of a preliminary investigation. A brief literature review on the influence of AAS parameters on other lignocellulosic biomasses was also carried out in order to provide an inspiration for optimization of the AAS process for digested manure fibers. Additionally, the main goals and frame of the AMMONOX project that is linked to this Thesis is presented. Based on this, a process configuration is suggested (Fig.8) for the incorporation of digested manure fibers and other lignocellulosic biomasses to liquid swine manure in biogas plants.

3.2. Related paper

Lymperatou, A., Gavala, H.N. Esbensen, K.H., Skiadas, I.V.
"AMMONOX: Ammonia for Enhancing Biogas Yield and Reducing NO_x—Analysis of Effects of Aqueous Ammonia Soaking on Manure Fibers", Waste and Biomass Valorization 2015 6: 449-457

3.3. Methodology

Previously obtained results were gathered from independently run Biochemical Methane Potential (BMP) tests of digested manure fibers, pretreated with AAS under different temperatures (20°C and 55°C), NH_3 concentrations (5, 10, 15, 20, 25 and 32 % w/w) and durations of AAS (1, 3 and 5 days) [139,167]. A matrix was formed and Principal Component Analysis (PCA) was used as an exploratory data tool for revealing groupings and correlations between the different AAS parameters and the resulting CH_4 yield. Two CH_4 yields were studied originating from the same experiments; the ultimate CH_4 yield was chosen in order to assess the effect of AAS parameters on the final biodegradability of digested fibers, and the cumulative CH_4 yield after ca. 18 days of digestion for evaluating the effects on the short-term CH_4 yield.

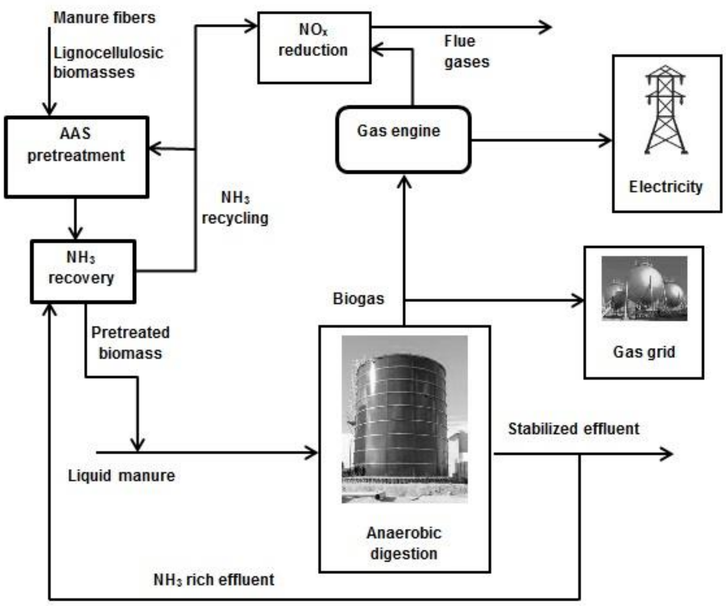

Figure 8 AMMONOX process. A suggestion for integrating the AAS pretreatment in manure-based anaerobic digestion processes (paper I)

3.4. Highlights

This preliminary study showed that, within the ranges tested, the temperature and the NH_3 concentration of AAS were the most influencing factors on the resulting ultimate CH_4 yield of pretreated digested manure fibers (Fig.9). The CH_4 yield was found to be independent of the AAS duration, indicating that durations shorter than 1 day should be tested in future optimization experiments. A strong negative correlation of the temperature with the CH_4 yield was detected, showing that heat application up to 55°C during AAS is not favorable for obtaining high CH_4 yields from this substrate. The NH_3 concentration of AAS was also strongly and negatively correlated to the CH_4 yield, i.e. the CH_4 yield was increased as NH_3 concentration was reduced within the range of concentrations tested. The same correlations were found for the cumulative CH_4 yield after 18 days of digestion, showing that the AAS parameters affected similarly the short-term CH_4 yield and the final biodegradability of digested manure fibers.

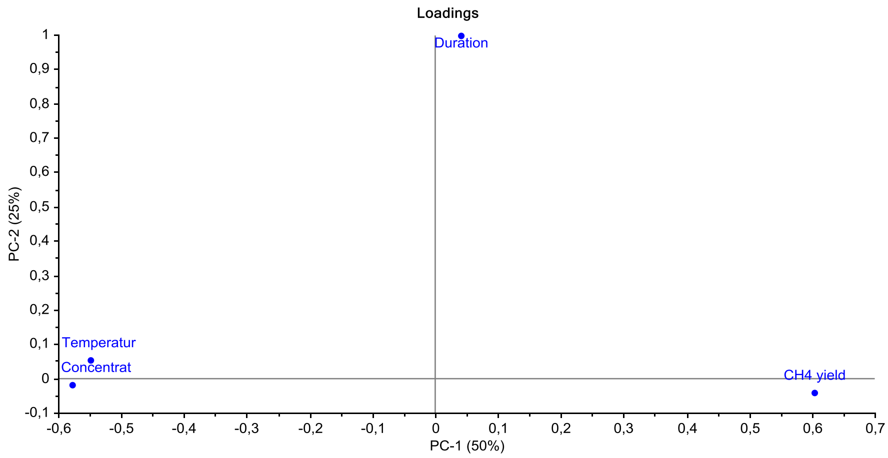

Figure 9 Loadings plot of the PCA showing the correlation of the three AAS parameters tested (temperature, NH_3 concentration and duration of AAS) with the ultimate CH_4 yield (paper I)

A brief literature review was also carried out in order to assess how AAS parameters affect other lignocellulosic biomasses for bioconversion processes. Based on this, contradictory findings have been reported mainly for the importance of the NH_3 concentration and the duration of AAS. However, the negative correlation of the temperature during AAS with the CH_4 yield of digested manure fibers, is in disagreement to most studies that report an increased efficiency of AAS at increasing temperatures [185,200,202–207]. As commented in section 1.4.3.2, this could be attributed to a higher lignin removal under high temperature. Thus, it can be hypothesized that lignin removal did not occur on digested manure fibers at 55°C, or that the formation of degradation products might have partially inhibited the anaerobic digestion process, leading to lower CH_4 yields than when treated at 20°C. This could explain the different observations as the whole pretreated fraction was used for the anaerobic digestion of digested manure fibers, while the rest of studies examined pretreated solids after washing with water.

Generally, the data set used for the PCA presented certain limitations. For example, not all AAS parameters were varied simultaneously in the different pretreatments of digested manure fibers. This limits the evaluation of interacting effects among AAS parameters, which have been reported to be significant in some studies [191]. For instance, if reducing further the duration of AAS the effect of increasing temperature might result to a positive correlation with the CH_4 yield and the duration of AAS might become a critical factor. Additionally, the effect of different levels of the solid-to-liquid (S:L) ratio, which has been found to be an important factor on the bioconversion of other biomasses [200], has not been tested on the CH_4 yield of digested manure fibers. Reducing the S:L ratio has been reported to result in an increase of delignification [200,208]. This could also explain why no lignin removal was observed in structural analyses of AAS-treated digested manure fibers [209].

Overall, the parameters chosen to study in combination to their respective ranges could highly influence their importance on an efficient AAS process. In regards to the influence of the AAS parameters on digested manure fibers, the importance of taking into consideration the S:L ratio as a potentially influencing factor and expanding the variation of duration levels were highlighted. Thus, it was concluded that future optimization experiments should initially investigate the effects of all four AAS parameters (temperature, NH_3 concentration, duration and S:L ratio), following an experimental design that will allow to detect interaction effects among them. Finally, an inherent problem with optimizing the AAS pretreatment of digested manure fibers is their highly variable composition due to co-digestion practices. Consequently, the efficiency of the process should be tested on digested manure fibers of different origin.

4. Optimization of AAS of Raw Swine Manure Fibers for maximizing the Methane Yield

4.1. Scope

The addition of pretreated raw manure fibers to swine manure is the most direct approach for simultaneously increasing the amounts of manure treated and addressing the low degradation rate of manure. AAS has been previously tested on swine manure fibers and was found to be very efficient on increasing the CH_4 yield by 178% as compared to non-pretreated fibers [167]. Nevertheless, only one set of conditions was tested involving a very high NH_3 concentration (32% w/w) [167], which would probably be prohibitive for an industrial application. A systematic evaluation of the influence of the different AAS parameters could lead to a better understanding of the potential and limitations of AAS on swine manure fibers, facilitating thus the process design prior to scaling up. Additionally, the evaluation of the compositional changes of optimally pretreated swine manure fibers could contribute to understanding the mechanism of AAS under optimal conditions for maximizing the CH_4 yield.

4.2.Related papers

Lymperatou, A., Gavala, H.N. Esbensen, K.H., Skiadas, I.V.
"Screening for the important variables of aqueous ammonia soaking as a pretreatment method for enhancing the methane production from swine manure fibers" Extended Abstract in Proceedings of 14th World Congress on Anaerobic Digestion, 2015, (Paper II)

Lymperatou, A., Gavala, H.N., Skiadas, I.V.
"Optimization of Aqueous Ammonia Soaking of manure fibers by Response Surface Methodology for unlocking the methane potential of swine manure" Paper III, (Accepted for publication in "Bioresource Technology").

4.3. Methodology

In this study, the influence of the different parameters of AAS of swine manure fibers was evaluated on the resulting CH_4 yield (both short-term and ultimate CH_4 yield). Initially a screening of the AAS parameters was carried out in order to identify the most influencing on the increase of the CH_4 yield of raw AAS-treated manure fibers. In the sequel, the effects of the most important parameters were further studied following the Response Surface Methodology (RSM). Empirical models were produced for expressing the influence of each parameter on the efficiency of AAS to increase the CH_4 yield of raw swine manure fibers.

The assessment of the CH_4 yield of pretreated swine manure fibers was based on BMP tests under mesophilic conditions (37°). In the screening experiments, different levels of temperature (20, 30 and 50°C), NH_3 concentration (0.6, 15, 32 % w/w), duration of AAS (12, 48, 120 days) and S:L ratio (1:3, 1:6, and 1:10 kg fibers/l reagent) were tested following a random design as described in [210]. The influence of the different AAS parameters on the resulting cumulative CH_4 yields was assessed through Partial Least Square Regression (PLS-R). Control experiments with non-pretreated manure fibers were run in parallel for assessing the increase of the CH_4 yield due to AAS.

In continuation, the effects of the most influencing factors as resulted from the screening experiments were further studied following Central Composite Designs (CCD). The optimal conditions of AAS for maximizing the CH_4 yield were assessed following the RSM. A two-step optimization was carried out. In the first step the parameters varied were the NH_3 concentration (0.9, 7, 16, 25, 31.1 % w/w), the duration of AAS (4.8, 28, 62, 96, 119.2 hours) and the S:L ratio (0.12, 0.16, 0.22, 0.28 and 0.32 kg fibers/l reagent) following a circumscribed CCD. The second step included the two interacting parameters towards the optimum region as resulted from the 1st step (NH_3 concentration 1, 4, 7% w/w and duration of 96, 120 and 144 hours), following a faced CCD. The main objective was to obtain information on how the different levels of the most influencing parameters of AAS affect the efficiency of the pretreatment on increasing the CH_4 yield of swine manure fibers. Empirical models expressing the CH_4 yield as a function of the AAS parameters were constructed and validated for facilitating the evaluation of different process configurations. Finally, the effect of AAS on the solubilization of COD was assessed and compositional analysis of the optimally AAS-treated manure fibers was performed in order to better understand the mechanism of AAS under these conditions.

4.4. Highlights

The Screening experiments showed a large variation of the cumulative CH_4 yield after 16 days of digestion. The increase of the CH_4 yield obtained from differently AAS-treated manure fibers as compared to untreated fibers varied from 46% to 197% depending on the conditions applied. This variation confirms the hypothesis that AAS presents a high potential for increasing the CH_4 yield of swine manure fibers, though this largely depends on the conditions applied. The S:L ratio, the NH_3 concentration and the duration of AAS appeared to significantly affect the CH_4 yield (Fig.10). On the contrary, the temperature of AAS, when varied from 20 to 50°C, was found to be the least influencing factor on the CH_4 yield of AAS-treated manure fibers (Fig.10). This could partly be due to the low maximum temperature applied. Nevertheless, higher values were considered not of interest as these would result to a high energy input for the implementation of AAS. In this line, heat application during AAS has rarely been studied on lignocellulosic biomasses for improving their CH_4 yield. Thus, the following optimization was based on the three most influencing factors while temperature was kept constant (20°C).

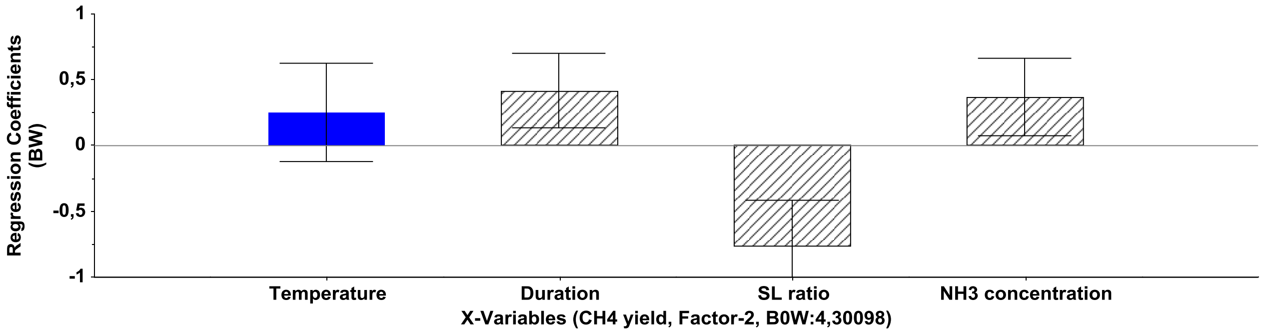

Figure 10 Centered and reduced regression coefficients with their 95% confidence intervals for the prediction of the CH_4 yield of swine manure fibers pretreated with AAS under different conditions (Screening experiments).

The first optimization step showed that the most important factor was the duration of AAS, and a strong interaction effect among the duration and the NH_3 concentration was detected. The effect of the S:L ratio was found to be independent of the rest of parameters, and to have a significant quadratic effect on the CH_4 yield, i.e. the lowest and highest values of the experimental region (0.16 and 0.28 kg/l) resulted in the highest CH_4 yields modelled. However, the highest yield experimentally obtained corresponded generally to the lowest S:L ratio tested. The Response Surface graph presented in Fig. 11a was constructed based on the model prediction for the short-term CH_4 yield as a function of the NH_3 concentration and the duration of AAS. The optimal conditions for maximizing the CH_4 yield (both short-term and ultimate) corresponded to 7% w/w NH_3, 96 hours of duration and 0.16 kg/l S:L ratio.

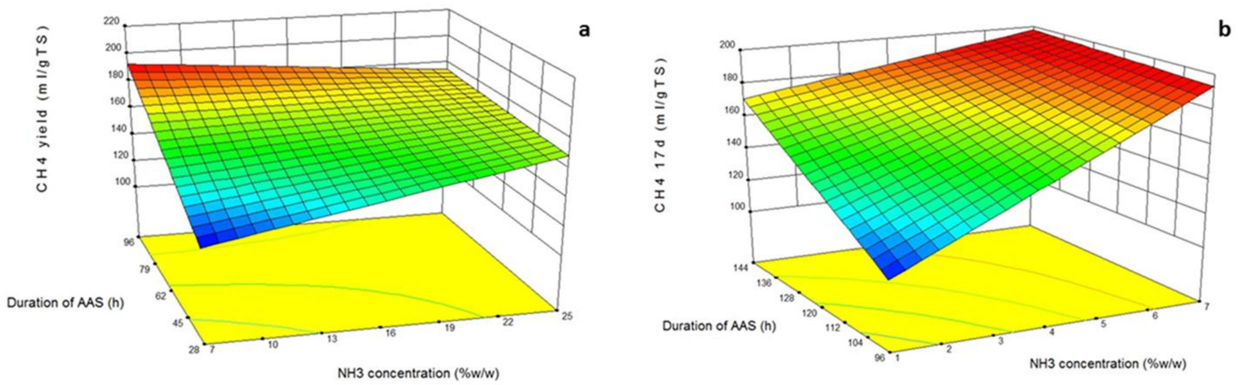

Figure 11 Response surface graphs of (a) 1st (cCCD) and (b) 2nd (fCCD) set of optimization experiments. The cumulative CH_4 yield of swine manure fibers is plotted as a function of the ammonia concentration and the duration of AAS. The S:L ratio of AAS is set constant at 0.16 kg fibers/l.

In the second step of optimization, focus was given on the interacting parameters (NH_3 concentration and duration) towards the optimum region, while the S:L ratio was kept constant at the optimum value (0.16 kg/l). Reducing the NH_3 concentration from 7% w/w down to 1% w/w made this factor critical for the increase of the CH_4 yield, resulting to be more influencing on the CH_4 yield than the duration. However the interaction of the two parameters (NH_3 concentration and duration) was significant in this experimental range as well, as a duration higher than 136 hours reduced the importance of the NH_3 concentration, showing that even at 5-6% w/w NH_3, high CH_4 yields can be obtained (Fig.11b). This clearly shows that the parameters found to be the most influencing, largely depend on the ranges chosen to study.

The optimal conditions of AAS based on the two-step optimization corresponded to 7% w/w, 96 hours of duration and 0.16 kg fibers/l. The average CH_4 yield obtained from all experiments under these conditions resulted to be 198.95 ± 9.49 ml/g TS, corresponding to a 244% increase of the short-term CH_4 yield as compared to the yield of the untreated manure fibers (57.89 ± 7.11 ml/g TS). AAS under optimal conditions reached 65.5% of the theoretical CH_4 yield in comparison to 12% obtained in the same digestion duration (17 days) by the untreated fibers. The fit of the models to the experimental data were generally satisfactory (R^2=0.84 for the 1st step optimization, and R^2=0.85 for the 2nd step of optimization). The ultimate CH_4 yields presented the same trends with the short-term CH_4 yields, as also found for the digested manure fibers (section 3.4).

Table 5 Pretreatments tested on swine manure fibers for improvement of methane yield under mesophilic conditions

Pretreatment method	BMP conditions*	Increase of CH_4 yield (%)	Reference
Alkaline treatment (NaOH)	32 °C, 30 d	13	[211]
Acidic treatment (HCl)	32 °C, 30 d	-10	[211]
Extrusion	35 °C, 28 d	27**	[212]
Thermal	40 °C, 56 d	171	[138]
Lignocellulolytic microbial consortium	37 °C, 60 d	55	[213]
Thermal	35 °C, 27 d	29	[214]
Thermal steam explosion	35.1 °C, 20 d	107	[215]
Aqueous Ammonia Soaking	37 °C, 17 d	244	This study

* Temperature at which the BMP tests took place followed by the number of days of BMP tests that the increase of CH_4 yield corresponds. **Average increase of different samples of pretreated manure fibers (both swine and cattle).

Swine manure fibers appear to respond very well to the AAS pretreatment in comparison to other pretreatments tested (Table 5). However, it is important to mention that most other pretreatments have not been optimized. Additionally, it has to be taken into account that the composition of manure fibers can differ depending on their origin and storage conditions as shown by [138], and thus manure fibers that result in highly different ultimate CH_4 yields when untreated, should be compared with precaution. Some studies report a similar ultimate CH_4 yield to the one obtained from swine manure fibers in this study [212,214,216], while in other studies significantly lower ultimate CH_4 yields were reported [138,213,215].

The solubilization of COD produced by AAS under different conditions was moderate, varying from to 5-15% of total COD. The soluble COD increased as the NH_3 concentration and the duration of AAS increased. During AAS, part of the reagent-N is bound to lignocellulose and this fraction has been reported to correspond generally to 2-5% of the dry matter (TS) of the biomass [202]. The composition analysis of the optimally AAS-treated manure fibers showed that only 0.92% TS of reagent-N was bound to the biomass. Additionally, significant solubilization of hemicellulose occurred during AAS (ca. 38%), while the cellulose and lignin fraction were not reduced. This indicates that the improved CH_4 yield probably resulted from the improved access of enzymes to carbohydrates (both cellulose and hemicellulose) as a result of the hemicellulose solubilization. The low temperature applied during AAS could partially explain why no lignin removal was observed in swine manure fibers, as lignin removal appears to be mostly promoted at high temperature levels, as also commented in sections 1.4.3.2 and 3.4. Nevertheless, in other studies moderate to high lignin removal has been reported on sunflower straw and rice straw when applying AAS at ambient temperature [168,217]. Additionally, in contrast to the findings of the present study, partial solubilization of cellulose has also been reported in different lignocellulosic biomasses treated under ambient temperature [168]. Thus, it appears that the mechanism of AAS depends largely on the biomass pretreated. A systematic study of AAS of different lignocellulosic biomasses under their respective optimal conditions could potentially shed some light on the mechanism of AAS depending on the specific composition of the biomass (e.g. type of lignin). This approach could also facilitate the detection of common characteristics of biomasses that best respond to AAS when aiming at the same response (e.g. CH_4 yield).

In conclusion, it was shown that AAS is highly efficient on improving the digestion of swine manure fibers. A relatively flexible range of pretreatment conditions resulting in high CH_4 yield was also observed. This is mainly due to the interaction among the NH_3 concentration and the duration of AAS detected in this study. Based on the empirical models constructed, in order to obtain 95% of the maximum increase of methane yield, 5-11% w/w NH_3 and 3.8-6 days of AAS duration can be applied. While the maturity of the manure fibers pretreated is expected to affect the cumulative CH_4 yields obtained, general trends on the increase of the CH_4 yield as a function of the AAS parameters can be derived from the empirical models produced in the present study. These could be used for orientating a techno-economic analysis and evaluating different AAS process configurations prior to scaling up.

5. Optimization of AAS of Wheat Straw for maximizing the Methane Yield

5.1. Scope

Agricultural straws could potentially be used as co-substrates for swine manure as they present a high C/N ratio that could alleviate the NH_3 inhibition of swine manure-based anaerobic digestion and boost the biogas production when efficiently pretreated. Wheat straw represents the most abundant agricultural residue in Europe [218] and thus it was considered to be a suitable alternative lignocellulosic biomass for swine manure-based anaerobic digestion. In this study, the influence of the AAS parameters on the CH_4 yield and COD solubilization of wheat straw pretreated at ambient temperature was evaluated. Additionally, the compositional changes on optimally pretreated wheat straw were studied, aiming at facilitating a better understanding of the AAS mechanism under optimal conditions.

5.2. Related Paper

Lymperatou, A., Gavala, H.N., Skiadas, I.V.
"Optimization of Aqueous Ammonia Soaking at ambient temperature for Enhancing the Methane Yield of Wheat Straw", Paper IV, Submitted.

5.3. Methodology

The AAS pretreatment of wheat straw has been previously applied and optimized at high temperature for biogas production [185]. In this study, the potential of AAS on wheat straw at ambient temperature (20°C) was evaluated in order to reduce the energy input needed and for assessing the possibility of integrating this biomass to a pretreatment process that initially operates on swine manure fibers. Additionally, as previously the anaerobic digestion of only the washed pretreated solid fraction of wheat straw was assessed [185], both the liquid and solid fractions were included in the digestion tests in this study, and focus was given to the degree of COD solubilization as a means to indicate how much of the organic matter is lost if only the solid fraction is sent to anaerobic digestion.

The assessment of the AAS parameters on the CH_4 yield of wheat straw was carried out by monitoring the CH_4 production of differently pretreated batches of wheat straw based on BMP tests. All pretreated BMP tests were run under mesophilic conditions (37°C). Prior to the AAS pretreatment, wheat straw was cut to 6 mm pieces in order to facilitate its handling. The conditions of AAS varied were the NH_3 concentration (1, 16.5, 32% w/w), the duration of AAS (1, 4 and 7 days) and the S:L ratio (50, 75 and 100 g straw/l reagent), following a faced CCD. The effect of the parameters on both the short-term CH_4 yield (after 17 days of digestion) and the ultimate CH_4 yield were assessed and optimized by RSM. The hydrolysis rate of differently AAS-treated wheat straw was also evaluated.

The hydrolysis rate is considered to be the limiting step in anaerobic digestion of lignocellulosic substrates and first order kinetics are often used for describing the hydrolysis process. The hydrolysis rates k of the BMP tests were calculated as described in [219] by applying equation 3 to each BMP test.

$$B = B_0 * (1 - e^{-kt}), \text{(eq.3)}$$

Where, B is the CH_4 yield after t days of duration and B_0 is the ultimate CH_4 yield. The criterion for choosing the duration t for each BMP was that the CH_4 yield had reached 65% of the ultimate yield, $B = 0.65 * B_0$.

5.4. Highlights

AAS at ambient temperature affected the hydrolysis rate of wheat straw in a positive way, and depending on the conditions applied it varied from 0.08 d^{-1} to 0.14 d^{-1} as compared to 0.07 d^{-1} of untreated wheat straw. Nevertheless, the highest hydrolysis rate did not correspond to the highest short-term CH_4 yield, as the final degradability of the biomass was also affected differently by the AAS conditions. A varied increased degree of COD solubilization was also observed (7-24% of total COD), which was found to be affected by the NH_3 concentration and the duration of AAS. The same trends were found for the solubilization of COD as for AAS-treated manure fibers, i.e. the soluble COD increased as the NH_3 concentration and the duration of AAS increased.

All three parameters of AAS were found to be highly influencing on both the short-term and ultimate CH_4 yield of pretreated wheat straw. Overall, AAS resulted in a short-term CH_4 yield between 223.40 ± 21.53 ml/g TS and 325.59 ± 6.96 ml/g TS under different AAS conditions, compared to 228.26 ± 17.90 ml/g TS of the untreated straw. Along the entire experimental region, the NH_3 concentration was identified as the most influencing factor, and similarly to swine manure fibers, the interaction among NH_3 concentration and duration was found to be highly significant. The S:L ratio was also found to have a strong quadratic effect on the CH_4 yield, as this was increased at the highest and lowest levels of S:L ratio. The optimal conditions according to the Response graph (Fig.12) corresponded to 18% w/w NH_3, 7 days of duration and 50 g straw/l. Under these conditions, a CH_4 yield of 325.87 ± 16.74 ml /g TS was achieved, corresponding to a 43% increase as compared to raw wheat straw. The anaerobic digestion of optimally pretreated wheat straw resulted in 73% of the theoretical CH_4 yield in only 17 days of digestion in comparison to the untreated straw where 52% was obtained. AAS pretreatment of wheat straw was found to be highly flexible in terms of ranges of operational parameters to be applied. Based on the empirical model constructed, a 95% of the maximum increase of methane yield can be obtained in 17 days of digestion when pretreating wheat straw with 7.3-29% w/w NH_3 concentration for 3.5-7 days with 50-60 g straw/l or 90-100g straw/l.

The conditions for maximizing the ultimate CH_4 yield of pretreated wheat straw were found to be even more flexible than for the short term CH_4 yield, as even under harsh conditions (32% w/w NH_3) and short duration of AAS (1 day) the biodegradability of wheat straw can be increased equally (red region in Fig.13a). In contrast to the short-term CH_4 yield, the degradability was found to be influenced also by an interaction among the S:L ratio and the NH_3 concentration (Fig.14) explaining thus the different optimal conditions for the ultimate CH_4 yield.

The compositional analysis of optimally pretreated wheat straw showed that hemicellulose was significantly solubilized (ca. 62%), while AAS has been reported to retain the hemicellulose fraction up to more than 80% [179,201,220]. Nevertheless, variable retention of hemicellulose has been observed by more researchers when applying AAS under different conditions [168,185,200,203,221,222]. The cellulose fraction was not solubilized by the pretreatment of wheat straw, similarly to swine manure fibers. On the contrary, the lignin fraction of wheat straw pretreated under optimal conditions and ambient temperature was partially removed (9%). Finally, the AAS pretreatment resulted to an increased total N content producing an undesirable effect by reducing significantly the C/N ratio from 63 to 28 and thus limiting the flexibility for co-digestion of wheat straw with swine manure.

In comparison to a previous optimization of NH_3 pretreatment of wheat straw involving heat application, AAS at ambient temperature improved similarly the anaerobic degradability of the biomass. Li et al. reported a 43% biogas yield increase obtained from the solid fraction of wheat straw treated under the optimal conditions suggested (51°C, 14.8% w/v NH_3, 27 hours of AAS) [185] which corresponds to the same CH_4 yield increase found by AAS at ambient conditions by digesting though both the liquid and solid fraction.

Numerous pretreatments have been tested on wheat straw up to know (Table 6). Overall, alkaline pretreatments appear to be among the most effective methods for improving the anaerobic digestion of lignocellulosic biomasses [131] and especially of wheat straw when compared to other pretreatments. The highest improvement of CH_4 yield reported in literature up to date corresponds to a NaOH based pretreatment, obtaining a 112% CH_4 yield increase [223]. However, the low CH_4 yield obtained for untreated wheat straw (78 ml/g VS) compared to the present and other studies [218] indicates that the raw wheat straw used was partially degraded. The degradation of the feedstock could influence significantly the efficiency of pretreatments, making more difficult a comparison among independent studies. An earlier study though, testing both AAS and NaOH pretreatment on wheat straw under the same conditions, showed that NaOH was more efficient on increasing the CH_4 yield of wheat straw [183].

Overall, it was found that AAS can increase significantly the biodegradation rate of wheat straw, producing up to 43% more CH_4 than the untreated straw within only 17 days of batch anaerobic digestion. Both the CH_4 production rate and ultimate yield were affected by the AAS pretreatment, and empirical models were constructed for expressing the effects of the AAS parameters on the resulting short-term (after 17 days of digestion) and ultimate CH_4 yield. In comparison to swine manure fibers, the optimal conditions of AAS for maximizing the short-term CH_4 yield of wheat straw are harsher (18% w/w NH_3 in contrast to 7% w/w NH_3 for manure fibers). However, a higher flexibility of the process configuration is permitted for wheat straw for achieving 95% of the highest increase of CH_4 yield (e.g. 7.3-29% w/w NH_3 in contrast to 5-11% w/w NH_3 for manure fibers). AAS under optimal conditions has affected the composition of wheat straw in a greater degree than swine manure fibers, by removing significantly higher amounts of the hemicellulose fraction and also removing part of the lignin. A more detailed compositional analysis is required for identifying the mechanism of AAS on different biomasses.

Table 6 Indicative results of pretreatments tested on wheat straw for improvement of CH_4 yield under mesophilic conditions

Pretreatment method	BMP conditions*	Increase of CH_4 yield (%)	Reference
NaOH pretreatment	35 °C, 40 d	112	[223]
Hydrothermal	37 °C, 60 d	20	[223]
Steam explosion	37 °C, 50 d	27	[224]
Thermal pretreatment	40 °C, 60 d	64	[225]
Alkaline-peroxide	35 °C, 30 d	7	[226]
Optimized Microwave pretreatment	37 °C, 60 d	28	[227]
Clostridium cellulolyticum	37 °C, 35 d	13	[228]
White rot fungi	37 °C, 30 d	28	[229]
Steam explosion	35 °C, 40 d	20	[230]
$Ca(OH)_2$ pretreatment	35 °C, 15 d	≈30	[231]
Optimized AAS with heat application	30 °C, 28 d	43**	[185]
Optimized AAS at ambient temperature	37 °C, 17 d (60 d)	43 (29)	This study

* Temperature at which the BMP tests took place followed by the duration of BMP tests in days

**Increase corresponds to biogas yield.

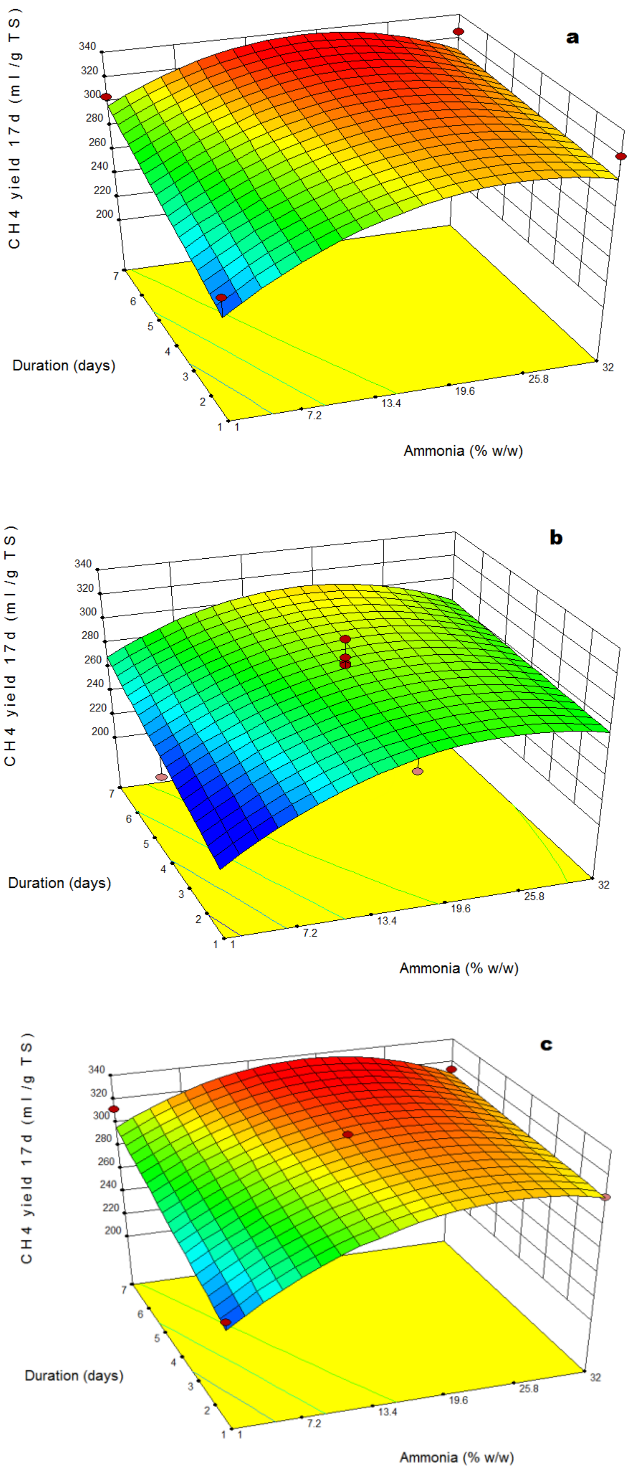

Figure 12 The surface represents the predicted CH_4 yield of AAS-treated wheat straw after 17 days of digestion as a function of the duration and NH_3 concentration of AAS at different S:L ratios: (a) 50 g/l, (b) 75 g/l, and (c) 100 g/l. Dots in figures correspond to the experimental points.

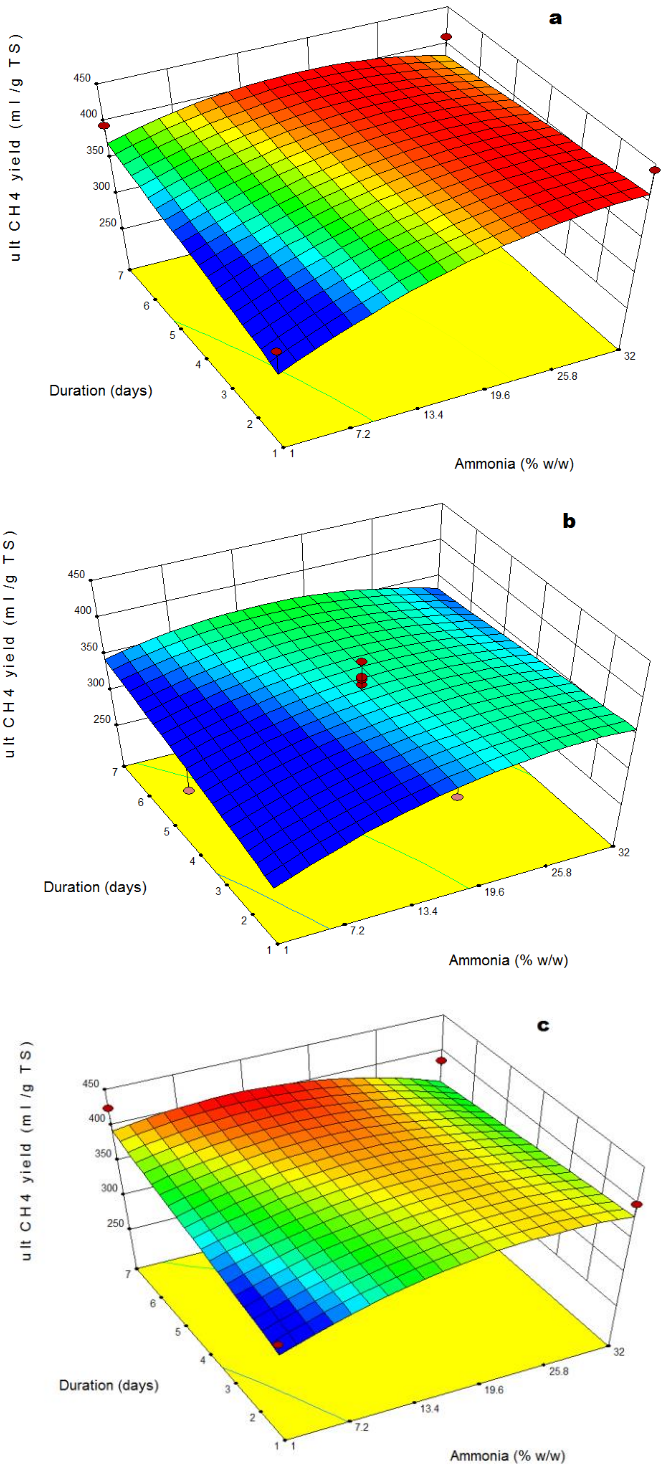

Figure 13 The predicted ultimate CH_4 yield of AAS-treated wheat straw is plotted as a function of the NH_3 concentration and the duration of AAS at different S:L ratios: (a) 50 g/l, (b) 75 g/l, and (c) 100 g/l. Dots in figures correspond to the experimental points.

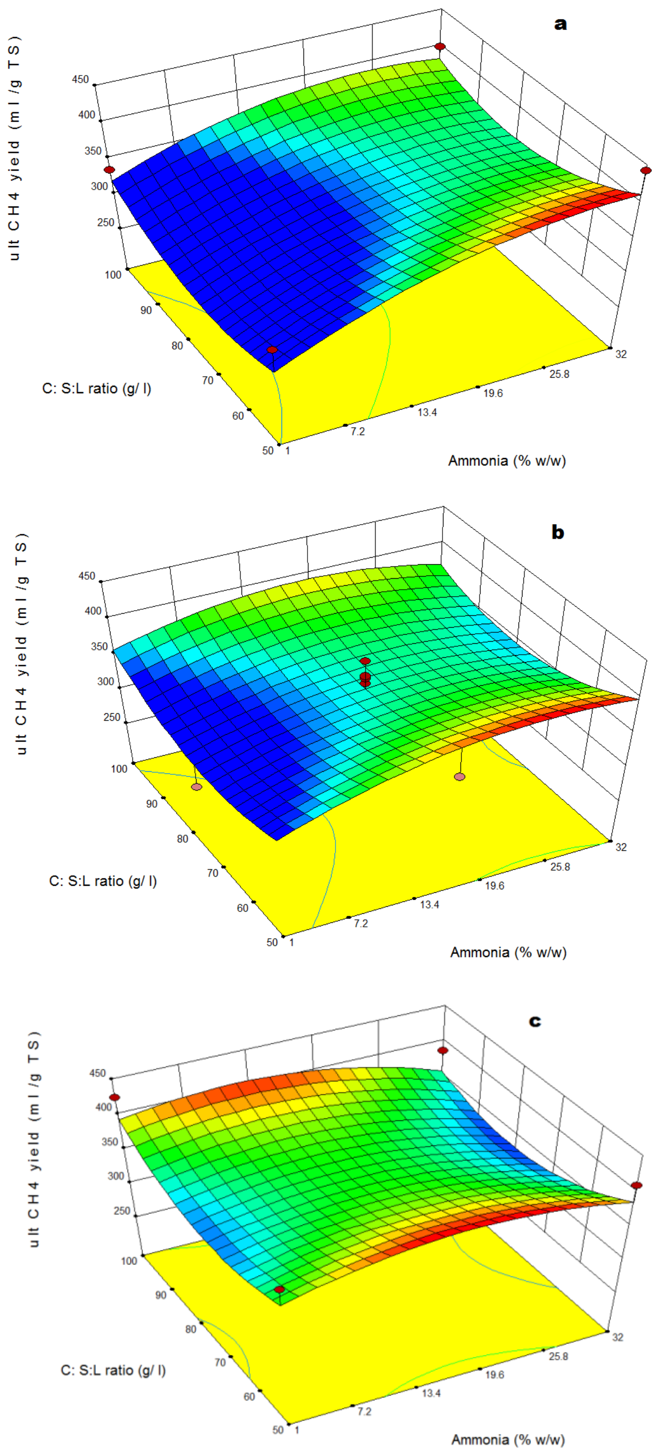

Figure 14 The predicted ultimate CH_4 yield of AAS-treated wheat straw is plotted as a function of the NH_3 concentration and the S:L ratio of AAS at different durations: (a) 1 day, (b) 4 days, and (c) 7 days. Dots in figures correspond to the experimental points.

6. Assessment of Performance of optimally AAS-treated raw swine manure fibers in continuous swine manure mono-digestion

6.1. Scope

Following the promising results obtained by AAS of raw swine manure fibers from batch experiments (Chapter 4), the performance of optimally AAS-treated fibers when enriched to swine manure was evaluated in continuous anaerobic digestion. In comparison to batch experiments, continuous mode anaerobic digestion can provide valuable information on the performance of the process in terms of biogas and CH_4 productivity and stability since these processes are closer to real applications. The main objectives of this study were to assess the biogas productivity and CH_4 yield of manure enriched with pretreated fibers, as well as the reduction efficiency of the major organic components, i.e. cellulose, hemicellulose, lignin, proteins and lipids, as compared to manure enriched with untreated manure fibers.

6.2. Related Paper

Lymperatou, A., Gavala, H.N., Skiadas, I.V.
"Effect of optimized Aqueous Ammonia Soaking of manure fibers on the continuous anaerobic digestion of swine manure", in progress, paper V.

6.3. Methodology

In order to assess the performance of optimally AAS-treated fibers when enriched to swine manure, three CSTR-type digesters (3 l active volume) were operated for 120-125 days. All processes were run at mesophilic conditions (37°C) with an HRT of 17-18 days and with organic loading rate of ca. 1g VS/l/d. The first digester was fed on swine manure alone in order to obtain an estimation of background productivity and yield of manure without fiber enrichment. In the sequel, the digested manure was used for inoculating two new digesters, one fed with swine manure enriched with optimally AAS-treated manure fibers (7% w/w, 4 days, 0.16 kg/l, AAS digester) and the other fed with swine manure enriched with non-pretreated fibers (NP digester). The feed of the mixture-based digesters was prepared based on a 2:1 ratio of swine manure to manure fibers (TS basis). NP fibers were diluted with tap water in order to obtain the same TS concentration of the influent as for the AAS digester. The comparison of the conversion efficiency of the major organic components was carried out through compositional analyses of the influents and effluents of the two mixture-based digesters. During the experiments the pH, soluble COD, TS, VS, total ammonia N (TAN) concentration, biogas and CH_4 production were monitored.

6.4. Highlights

The addition of manure fibers (both NP and AAS-treated) to liquid manure resulted in an increase of the carbohydrate fraction in the mixture-based digesters. In both processes where manure fibers were added to swine manure (NP and AAS digester) the initially moderate TAN concentration of manure (2.73 g/l) was further reduced (1.82 g/l and 2.04 g/l for the NP and AAS digester respectively), reducing thus the risk for NH_3 inhibition. Generally though, no inhibition was observed in all three processes, confirmed by the low VFA concentrations in the digesters (0.19-0.26 g/l).

The AAS digester presented an increased biogas and CH_4 productivity in comparison to the control digester fed only with raw manure (12% and 7% respectively). The NP digester, besides having the lowest TAN and free NH_3 concentration among all processes, presented a lower productivity than the control digester (-5% and -11% biogas and CH_4 productivity in comparison to the control digester). Thus, the low biodegradability of the added fibers did not improve manure mono-digestion regardless the improved TAN concentration. In a previous study, Møller et al. operated a digester fed with swine manure enriched with NP manure fibers, and the share of fibers was gradually increased up to 60% (corresponding to a 15.5% TS loading) [105]. The resulting productivity was higher in the high solids digester while the CH_4 yield proved to be less than in the low solids digester. This was partly attributed to the higher NH_3 concentration in the high solids digester as a result of the higher TAN content of fibers in comparison to manure, producing inhibition of the process. However, our study showed that even when the added manure fibers present a lower TAN concentration than manure, the biogas production may not be improved as the degradability of the fibers is a more decisive factor for improving manure mono-digestion.

The efficiency of the pretreatment on increasing the energy recovery of manure fibers could be evaluated by comparing the performance of the mixture-based digesters. Overall, the AAS digester presented an improved performance, increasing the biogas and CH_4 productivities by 17% and 20% respectively when compared to the NP digester. By assuming that the CH_4 yield of swine manure contributed equally to the mixture-based digesters (204ml/g TS_{fed}), and taking into account the manure-to-fibers TS feed ratio, it was calculated that the CH_4 yield of the AAS manure fibers corresponded to 235.7 ml/g TS_{fed} while the CH_4 yield of the NP fibers to 59.7 ml/g TS_{fed}. This corresponds to a 295% increase of the CH_4 yield obtained by AAS on manure fibers at continuous digestion with an HRT of 18 days, which is higher than found at batch experiments (section 4.4).

Table 7 Removal of major components in continuous anaerobic digestion of manure enriched with non-pretreated fibers (NP digester) and with optimally AAS-treated manure fibers (AAS digester)

Component	NP digester			AAS digester		
	Influent (g/kg)	Effluent (g/kg)	% reduction	Influent (g/kg)	Effluent (g/kg)	% reduction
VS	21.87 ± 0.53	11.85 ± 1.93	45.8	20.32 ± 1.96	10.02 ± 2.16	50.7
Cellulose	4.76 ± 0.00[a]	2.74 ± 0.02	42.6	4.53 ± 0.11[a]	1.80 ± 0.11	60.3
Hemicellulose	3.83 ± 0.01[a]	1.76 ± 0.02	54.1	2.81 ± 0.01[a]	0.98 ± 0.06	65.3
Proteins	6.21 ± 0.79[a]	3.70 ± 0.03	40.5	6.25 ± 1.20[a]	3.27 ± 0.03	47.7
Lipids	2.37 ± 0.18[a]	1.52 ± 0.13	35.6	2.01 ± 0.01	1. 11 ± 0.19	44.6
Lignin	4.92 ± 0.36[a]	3.03 ± 0.00	38.5	4.61 ± 0.35[a]	2.39 ± 0.10	48.2

[a] Estimated through mass balance

The improved bioconversion of pretreated manure fibers was also evidenced by the increased reduction of the major organic groups of the influents (Table 7). The main difference among the digesters was observed in the carbohydrate content of the feeds, and especially in the reduction efficiency of cellulose. A 60.3% reduction of cellulose was achieved from the AAS digester in comparison to only 42.6% from the NP digester (Table 7), corresponding to a 42% improved reduction efficiency of cellulose. Interestingly, the rest of components, including proteins, lipids and lignin were also reduced in a greater degree in the AAS digester, indicating that AAS affected to a certain extent also the reduction of these fractions (Table 7). As a result of the significantly reduced cellulose content in the effluent of the AAS digester, the cellulose/lignin ratio, which has been suggested as an indicator of maturity of digestates [62], was significantly reduced from 0.98 to 0.78 in the AAS digester in comparison to a reduction from 0.97 to 0.90 in the NP digester, (Fig.15).

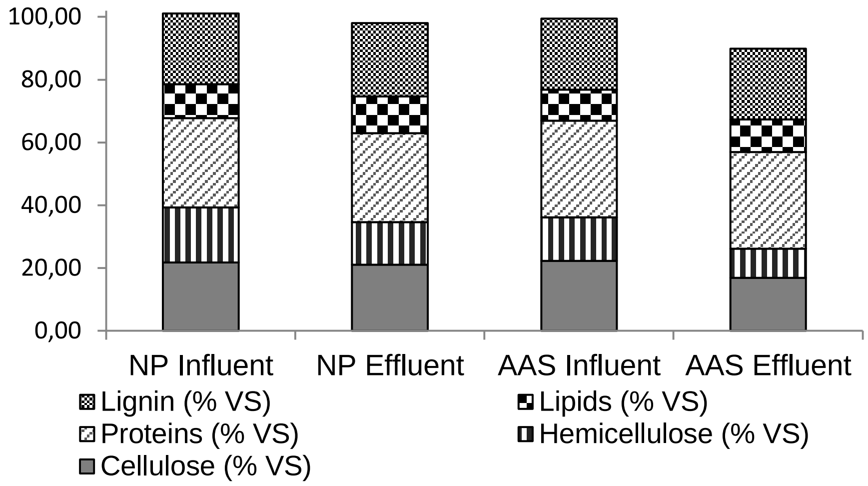

Figure 15 Composition of influents and effluents of NP and AAS digesters expressed in % of VS.

Overall, the efficiency of optimal AAS on improving the biogas and CH_4 production of swine manure fibers when added to swine manure was confirmed on continuous anaerobic digestion tests. Additionally, AAS significantly increased the reduction of all organic components, observing an especially significant reduction of the cellulosic fraction. The necessity to investigate an increasing share of pretreated fibers in the feed was highlighted in order to evaluate the stability of a high solids process, which could lead to a significant increase of the amounts of manure treated anaerobically.

7. Overall Conclusions

The main goal of this PhD study was to evaluate the potential of Aqueous Ammonia Soaking (AAS) under different conditions as a pretreatment method for increasing the CH_4 yield of lignocellulosic biomasses, in order to improve swine manure-based biogas production. The efficiency and economic feasibility of AAS greatly depends on the conditions applied, and thus assessing the influence of the AAS parameters on the resulting CH_4 yield is necessary prior to designing a process configuration.

Initially, a preliminary study was conducted for evaluating the influence of the different AAS parameters on the increase of the CH_4 yield of digested manure fibers. This was based on an exploration of results obtained from previous batch anaerobic digestion experiments of digested manure fibers treated under different AAS conditions (temperature, NH_3 concentration and duration). This study showed that both an increase of the temperature (up to 55°C) and the NH_3 concentration (up to 32% w/w) during AAS produce a decrease of the CH_4 yield of digested manure fibers within the ranges of the parameters tested. On the contrary, the duration of AAS (from 1 to 5 days) was found to be insignificant to the efficiency of the pretreatment. However, the limitations of the data set studied, indicate that in order to obtain a full view of the effects of the AAS parameters on the CH_4 yield of digested manure fibers and optimize the pretreatment, an experimental design enabling to detect interaction effects among the AAS parameters is necessary. Additionally, the S:L ratio has to be taken into account, as it could potentially influence significantly the mechanism of AAS, and thus the CH_4 yield of digested manure fibers. Based on a brief literature review on the influence of the different AAS parameters on improving the biodegradability of lignocellulosic biomasses, it was concluded that no general conclusions can be drawn on the influence and importance of the different AAS parameters from studies that evaluate different ranges of the AAS parameters on different biomasses.

Increasing the CH_4 yield of (raw) swine manure fibers is the most direct way to improve swine manure anaerobic digestion. For this reason, this biomass was extensively studied in this PhD Thesis. A systematic study was carried out, including screening and optimization experiments, where batch anaerobic digestion tests of swine manure fibers treated under different AAS conditions were carried out and the CH_4 yield was monitored. Screening experiments showed that the most influencing factors of AAS on the increase of the CH_4 yield of swine manure fibers, are the NH_3 concentration, the duration of AAS and the solid-to-liquid (S:L) ratio during AAS. On the contrary, the temperature during AAS was found to be the least important (within the ranges tested). Thus, the three most influencing factors were further studied, aiming at evaluating how these affect the CH_4 yield of pretreated fibers and how flexible these could be for ensuring high CH_4 yields. The optimization experiments showed that the NH_3 concentration and the duration of AAS are highly interactive, leading to mild optimal conditions for maximizing the CH_4 yield (7% w/w NH_3 for 4 days). On the other hand, the S:L ratio was found to have a strong effect on the CH_4 yield and no interaction effects with the rest of parameters. This finding shows that the biomass concentration during AAS can be adjusted independently of the rest of AAS parameters providing with more flexibility the process configuration. The S:L ratio has a quadratic effect on the increase of the CH_4 yield of manure fibers, i.e. higher CH_4 yields are obtained at low (160 g/l) and at high S:L ratios (280 g/l), while at middle values (220 g fibers/l reagent) the poorest increase of the CH_4 yield is obtained. Based on the experiments run, empirical models were constructed able to predict the increase of the CH_4 yield as a function of the levels of the AAS parameters. Based on these models, in order to ensure a 95% of the maximum increase of the CH_4 yield (244%), relatively mild conditions (5-11% w/w NH_3, 3.8-6 days) and low S:L ratios (160-170 g/l) are required.

The potential of AAS under different conditions on increasing the CH_4 yield of wheat straw was also investigated, as it is an abundant agricultural residue, and its co-digestion with swine manure is an alternative way to improve manure-based biogas production. The influence of three AAS parameters (NH_3 concentration, duration of AAS and S:L ratio) on the CH_4 yield of wheat straw were investigated in batch anaerobic digestion, aiming at a low energy input pretreatment process at ambient temperature (20°C). Optimization experiments showed that the parameters studied have a similar influence on the short-term CH_4 yield of wheat straw (after 17 days of digestion) as on the CH_4 yield of manure fibers, i.e. the resulting CH_4 yield can be expressed by a strong interaction among the NH_3 concentration and the duration of AAS, along with a quadratic effect of the S:L ratio. Nevertheless, the NH_3 concentration itself had a great direct impact on the resulting CH_4 yield of wheat straw. The optimal conditions for maximizing the short-term CH_4 yield of wheat straw were defined, and correspond to harsher conditions than for swine manure fibers (18% w/w NH_3, 7 days, 50 or 100 g straw/l reagent). Under these conditions, a maximum increase of 43% of the short-term CH_4 yield can be achieved. However, the conditions of AAS for ensuring a 95% of maximum increase of the CH_4 yield are very flexible (7.3-29% w/w, 3.5-7 days, 50-60 g/l or 95-100 g/l). Additionally, it was found that in order to maximize the ultimate CH_4 yield of wheat straw (when no further CH_4 is produced based on batch experiments) the conditions applied can also be harsher and of lower duration (32% w/w, 1 day, 50 g/l).

In order to evaluate the mechanism of AAS on the optimally pretreated biomasses that leads to the maximum CH_4 yield, the biomasses were analyzed for assessing the compositional changes that occur under their respective optimal AAS conditions. The results obtained, showed that only the hemicellulose fraction of swine manure fibers is solubilized (37%), while moderate lignin removal (9%) and significantly high solubilization of hemicellulose (62%) occurs on wheat straw when pretreated under optimal conditions. The analysis of the soluble fraction showed that part of the hemicellulose is recovered mainly in the form of oligosaccharides, while part of it is converted to other byproducts not detected.

Following the optimization of AAS of swine manure fibers and the promising results obtained in batch experiments, focus was given on the performance of AAS-treated manure fibers in continuous manure-based anaerobic digestion. A comparison among two continuous anaerobic digesters was performed, one fed with swine manure mixed with optimally AAS-treated swine manure fibers and one fed with swine manure mixed with untreated manure fibers. The aim of this study was to evaluate the effects of the optimized AAS in continuous anaerobic digestion, in regards to the biogas productivity, the CH_4 yield and the reduction efficiency of the major organic components of manure fibers (carbohydrates, proteins, lipids and lignin). Based on the results obtained, a 17% and 38% increase of the biogas productivity and CH_4 yield respectively can be obtained when manure fibers are treated with AAS under optimal conditions. The addition of optimally AAS-treated fibers resulted in an improved reduction efficiency of all major organic components of swine manure fibers, being cellulose the fraction mostly affected (42% increased reduction efficiency due to optimal AAS).

Overall, it was concluded that AAS is an efficient and relatively flexible process for increasing significantly the CH_4 yield of the lignocellulosic biomasses tested. Generally, both the degradation rate and ultimate degradability of the biomasses were improved. Ambient temperature (20°C) during AAS was found to be sufficient for increasing the CH_4 yield of both raw and digested manure fibers, permitting for a low energy process. While the conditions for maximum increase of the CH_4 yield are harsher for wheat straw than for manure fibers and optimal conditions differ, common effects of the AAS parameters on the CH_4 yield (interaction of NH_3 concentration and duration, and quadratic effect of S:L ratio) of the pretreated biomasses exist. Additionally, it was shown that optimal AAS improves the reduction efficiency of carbohydrates, proteins, lignin and lipids.

8. Future Perspectives

This PhD study showed that AAS under optimal conditions followed by ammonia removal has the potential to improve significantly the CH_4 yield of swine manure fibers, providing thus a potential solution to the limitations of swine manure mono-digestion. Additionally, the assessment of the influence of the AAS parameters on the CH_4 yield obtained of pretreated wheat straw showed that a high flexibility can be allowed on the configuration of the process for increasing the degradability of this feedstock. A lot of research though should still take place in order to improve our understanding on the process limitations and accelerate the integration of the AAS pretreatment to manure-based anaerobic digestion processes.

An important aspect of the process proposed in this Phd study includes the flexibility of application to biomasses of different origin. Especially manure fibers may present a varying composition depending on the feed of the animals, the manure management practices (bedding material used), the separation technology used, the storage conditions and its duration. Thus, the effect of AAS should be tested on differently produced manure fibers in order to assess the effect of AAS and determine which management practices will not reduce the potential of the process proposed. Additionally, as high ammonia concentration is a common characteristic among different manures, by extending the application of AAS followed by ammonia removal to manure fibers produced by other animals (e.g. cattle, chicken), a common approach for improving manure mono-digestion could be formulated. This could also facilitate the optimization of AAS of digested manure fibers that are per se of highly variable composition due to co-digestion practices. In the same line, in order to expand the applicability of the proposed process, these investigations should include also the evaluation of AAS-treated manure fibers under thermophilic conditions of anaerobic digestion.

The main goal of this study was to investigate the flexibility of the AAS process on increasing the methane yield of lignocellulosic biomasses for improving manure-based anaerobic digestion and ultimately for increasing the amounts of manure processed. While focus was given to the energy recovery from manure-based anaerobic digestion, the driving force of this PhD study lies on reducing the negative environmental impacts of swine manure management. Thus an environmental Life Cycle Assessment (LCA) would be necessary for assessing whether such a process would be sustainable. The use of ammonia might seem prohibitive when considering its environmental impact. Though if ammonia is efficiently recycled and appropriate measures are taken for avoiding losses, this could even result to a better control of ammonia emissions from both swine manure and anaerobic digestates. Furthermore, LCA may indicate which parts of the process result in the highest environmental impact and thus direct further research for improvement of the process.

Significant research could still be done on understanding better the mechanism of AAS on different lignocellulosic biomasses. Based on this study and in combination with previous research where AAS is evaluated on different biomasses at ambient temperature, a large variation on the compositional changes due to AAS has been evidenced. For instance, it appears that the lignin fraction of some biomasses is removed even at ambient temperature while this is not a general observation. The assessment of the specific characteristics of lignocellulosic biomasses that result in lignin removal or cellulose and hemicellulose solubilization as a result of the conditions of AAS could shed some light on the mechanism of AAS. In turn, this could indicate common characteristics of the biomasses that best respond to this pretreatment.

The solubilization of organic components like lignin and hemicellulose points to the need of investigating the liquid fractions of AAS pretreated biomasses for the formation of by-products and their fate during anaerobic digestion. Similarly, it is known that when ammonia reacts with the biomass, part of the N is bound to the organic matter and thus not recovered during ammonia removal. From a process point of view this might not result in a problem for recovering and reusing ammonia, as part of the effluent can be used for fulfilling the chemical requirements. Nevertheless, identification of these N complexes could improve our understanding of the AAS mechanism and the fate of these compounds during anaerobic digestion.

In order to significantly improve the biogas production of swine manure by addition of AAS-treated swine manure fibers, the high dilution of the influent should be addressed. In this study it was not possible to increase the dry matter of the influent above 3% due to lab-scale limitations, while the pumps of large scale digesters can allow influents up to ca. 12% dry matter. Consequently it is important to test how the process is affected from a stability and productivity point of view when higher addition of AAS-treated biomasses takes place in a continuous process. Although a further boosting of the biogas production could be expected, this should be tested first at pilot scale in order to ensure that the high solids system is stable.

In the same line, the possibility of digesting only the solid fraction should be investigated in the future, as this would significantly reduce the volume of the ammonia recovery step and increase the solids concentration of the pretreated biomass to digest, possibly resulting to a more economical process. In this study, the solubilization of COD was investigated under different conditions of AAS, for providing an estimation of the extent of solubilization of the solid matrix of the biomass and thus of the amount lost in the liquid fraction. While a more in depth characterization of the liquid fraction of pretreated biomasses is needed, the models produced for indicating the solubilization of COD can be helpful for orientating the process configuration as regards to the fractions that are finally sent for anaerobic digestion (whole fraction or only solid fraction).

Finally, AAS is necessarily coupled to an ammonia removal step and ideally to an ammonia recovery step. While the removal could be expected to be relatively easy to implement as some technologies already exist (e.g. ammonia stripping), these technologies have not been tested on concentrations as high as used in AAS. Thus, both practical and economic constraints might occur that need to be evaluated in the future. In the same line, a techno-economic analysis of the proposed process should take place, as this could help to identify the flexibility for implementation of different ammonia recovery methods. This PhD study resulted in the construction of empirical models that predict the effects of the different parameters of AAS on the CH_4 yield of the studied biomasses. These models could be used to indicate the increase of CH_4 yield expected under different configurations and thus assist the techno-economic analysis of the process.

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9. Papers

Paper I:

"AMMONOX: Ammonia for Enhancing Biogas Yield and Reducing NOx—Analysis of Effects of Aqueous Ammonia Soaking on Manure Fibers"

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ORIGINAL PAPER

AMMONOX: Ammonia for Enhancing Biogas Yield and Reducing NO_x—Analysis of Effects of Aqueous Ammonia Soaking on Manure Fibers

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Abstract Laboratory experiments have shown that aqueous ammonia soaking (AAS) is a promising treatment for increasing the methane yield of the solid fraction of manure (fibers). AMMONOX is a new concept based on the sustainable use of ammonia for enhancing biogas production at biogas plants digesting manure. The proposed process is based on an optimized AAS treatment of manure fibers in combination with an efficient ammonia recovery step. The enhancement of biogas production is achieved by enriching manure with AAS-treated fibers, or other lignocellulosic residues, while the ammonia recovered can be used for fulfilling the needs of the treatment itself. Excess of ammonia could be produced when ammonia is recovered from both the treated fibers and the digester effluent, which could be used for the reduction of NO_x in biogas-based electricity generation by gas turbines. In this survey study, the importance of different factors affecting the performance of AAS of digested manure fibers was investigated in order to conclude on which variables to optimize. Principal component analysis of the present data was used for a preliminary analysis of effects. The temperature and the ammonia concentration during AAS were the most influencing variables in terms of methane yield under the conditions tested. Further experiments should be conducted in order to investigate the effect of shorter AAS duration than the ones tested (lower than 24 h) and for assessing the importance of the solid-to-liquid ratio in the treatment mixture; the follow-up campaign should be optimized with respect to possible interactions/correlated experimental factor effects.

Keywords Biogas · Pretreatment · Aqueous ammonia soaking · Methane yield · Manure fibers · Principal component analysis

Introduction

Livestock manure has been pointed out as one of the most important agricultural sources of environmental pollution. Manure is rich in valuable nutrients for plant growth such as nitrogen and phosphorus, which makes its use as a crop fertilizer and soil amendment a common practice in many countries. However, a large part of these nutrients are susceptible to loss to the environment through leaching, run-off or volatilization [1]. Due to the large contribution of manure to ammonia, greenhouse gas emissions and water pollution, concerns about its management have increased, and many regions in Europe that have an intensive livestock production, are struggling to find solutions in order to comply with the disposal limits stipulated in environmental legislation (91/676/EEC) [2].

Anaerobic digestion is one of the most attractive solutions for manure management, as it provides stabilization of nutrients as well as reduction of gas and odor emission. Moreover, the naturally produced methane is captured and can be used in the form of biogas as an alternative energy source. Financially the digestion of solely manure is however, a non-feasible process due to its low content in easily digestible organic matter [3]. This is because the easily digestible part of the

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animal feed has already been utilized in the animal digestion; thus manure contains a recalcitrant lignocellulosic concentrated part [4] mixed with washing water and other lignocellulosic biomasses, such as straw, that are used for bedding materials [5]. Consequently, manure-based biogas plants are forced to search for easily digestible organic materials, such as food waste, to be used for co-digesting with manure to ensure a cost-efficient process. Unfortunately, as the demand for these extra materials increases, their availability remains very limited, presenting biogas plants with a new problem [3].

As an alternative to co-digesting, pretreating the solid fraction (fibers) of manure could present a solution to its recalcitrant nature and enhance the methane yield when it is digested. Different manure separation techniques have been proposed [6], and several biogas plants are already equipped with a decanter centrifuge for this purpose [7]. The separation of the two fractions (liquid and solid) makes it possible to increase the final dry matter content of the material to digest, leading this way to an even higher biogas production per mass unit [8]. Various researchers have used different approaches for pretreating manure fibers in order to make them more easily degradable and increase the methane yield. According to a recent survey [9], aqueous ammonia soaking (AAS) has achieved the highest increase of methane yield of manure fibers, both of raw (separated from manure) and digested (separated from the effluent after a first digestion).

Ammonia has been used in the past for increasing the digestibility of straw for ruminants feeding [10], and only recently it has been used for improving the biofuels production. AAS is a very simple pretreatment that has so far been tested on some lignocellulosic biomasses for increasing ethanol production, and has recently captured the interest of researchers for biogas production. Apart from manure fibers, other biomasses that have been tested for this purpose include switchgrass [11], wheat straw [12–14], corn straw [15], rice straw [16] *miscanthus* and willow [13]. During the AAS pretreatment, the biomass in question is mixed with a water solution of ammonia and left over a certain period of time at mild temperatures (less than 90 °C). Subsequently, the ammonia is removed and the biomass can be used for digestion.

Despite the efficiency of AAS on increasing the methane yield of manure fibers, a significant variation of the performance of AAS under the conditions tested up to now has been observed. Jurado et al. [17] have performed AAS of digested manure fibers under different levels of temperature and duration achieving an increase of methane yield between 30 and 80 %. Further experiments with lower ammonia concentrations led to a higher increase of methane yield (up to 205 %) [9]. Batch experiments for the determination of the methane potential of AAS-pretreated raw manure fibers showed that AAS pretreatment increased the methane yield by 178 % compared to non-pretreated fibers [17]. These results clearly show that AAS has a great potential for increasing the methane yield of manure fibers. Still, optimization of the most important parameters of AAS affecting the methane yield is necessary.

AMMONOX is a research project that aims to increase the efficiency of manure-based biogas production under an integrated process. The idea consists of an optimized ammonia treatment of manure fibers (or of other lignocellulosic biomasses) in combination with a successful ammonia recovery step (Fig. 1). As illustrated in Fig. 1, the manure fibers are first treated with a solution of aqueous ammonia under the conditions found to be optimal. Afterwards, the pretreatment mixture passes through a second process (Fig. 1) where the ammonia is removed until reaching a concentration low enough to avoid inhibition of the biological processes. After this step, the pretreated fibers are inserted to the digestion tank together with raw manure. The removal of ammonia is a relatively easy process due to its high volatility, thus providing an extra advantage of this pretreatment. This allows recovering ammonia and recycling it for fulfilling the chemical requirements of AAS, resulting in no extra consumption of chemicals. Additionally, as shown in Fig. 1, an excess of ammonia could be produced when the ammonia removal step includes both AAS-treated fibers and effluent from the anaerobic digestion step. This excess of ammonia can be used for the catalytic reduction of the NO_x produced, when the biogas is used for electricity generation by gas engines [18]. This is a commercially available technology and the ammonia required can be either in a gaseous form or in an aqueous solution [19].

High concentrations of ammonia are known to be inhibitory for the anaerobic digestion process, affecting mostly the methanogenesis step [20]. Thus after the AAS pretreatment, an ammonia removal step is essential for ensuring a stable process. Furthermore, the recovery of ammonia is of high importance for reducing the cost of the process as the ammonia can be recycled. Swine manure is often rich in ammonia, and during the anaerobic digestion process, degradation of proteins also takes place resulting to an increased ammonia concentration in the effluent. Some biogas plants are already equipped with ammonia- stripping technologies either for treating raw manure or for the post-treatment of the digestate prior to final disposal [7]. Nevertheless, the adequacy of ammonia-stripping of streams with such high ammonia concentrations as used in the AAS treatment has not been investigated yet. Moreover, the recovery of ammonia by stripping technology is achieved by means of acids, resulting to ammonium salts as final products. Therefore, modifications of stripping technology as well as additional methods should be considered for an efficient recovery of pure ammonia.

In summary, the three objectives of AMMONOX are:

- The optimization of the most important parameters of AAS affecting the methane yield of manure fibers, and

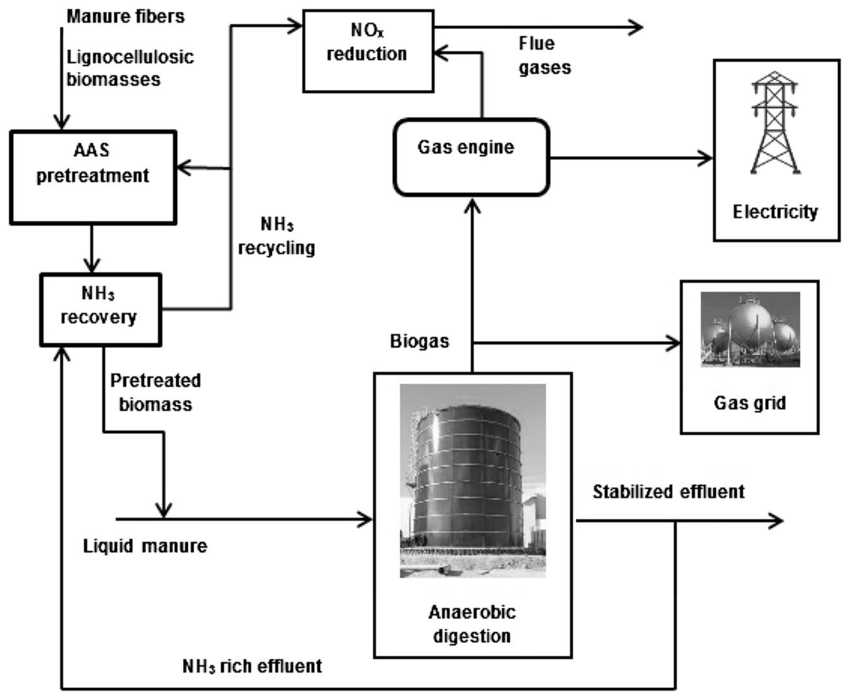

Fig. 1 General scheme of the AMMONOX concept for an enhanced manure-based biogas production in a sustainable way

of other lignocellulosic biomasses, for an enhanced manure-based biogas production,

- The identification and application of a successful ammonia removal technology that will permit to recycle the ammonia needed for AAS, and
- The use of the excess of ammonia recovered for the catalytic reduction of NO_x.

The focus of this study lays on the first objective of the AMMONOX process, which is considered to be crucial for identifying the adequate ammonia recovery technology and assessing the surplus of ammonia produced for the NO_x reduction. In order to develop a process for optimizing the AAS treatment in terms of maximum methane yield, some preliminary analyses were conducted and the results are presented in this study. A data exploration method was used for uncovering the possibly hidden information from the experimental data obtained so far, during studies of the effect that different conditions of AAS treatment of digested manure fibers had on methane yield.

Materials and Methods

Data Set

All the experimental data used in this analysis originate from research at Aalborg University and are published in Jurado et al. [17] and Mirtsou-Xanthopoulou et al. [9]. The data consist of CH_4 yields of digested swine manure fibers pretreated with AAS under two different temperatures (22 and 55 °C), three different pretreatment durations (1, 3 and 5 days) and six different NH_3 concentrations of the reagent (5, 10, 15, 20, 25 and 32 % w/w). All pretreatment mixtures were further used for experiments after a distillation step where the NH_3 concentration was reduced to below-inhibition levels [9]. Two data sets were formed for the data exploration analysis, both originating from the same experiments. The first data set, presented in Table 1, concerned ultimate CH_4 yields (CH_4 yield when no further gas production was detected, usually more than 30 days) of biochemical methane potential (BMP) tests; and the second data set concerned the CH_4 yields after approximately 18 days from the same experiments. The purpose of analyzing the two different data sets was to assess the effect of the AAS parameters on the short term CH_4 yield and on the ultimate CH_4 yield. Both data matrixes were constructed by 54 rows, corresponding to the total amount of experiments (including triplicates), and 4 columns, corresponding to the pretreatment variables (temperature, duration and NH_3 concentration of the reagent) and to the CH_4 yield obtained under these conditions.

Principal Component Analysis

Principal component analysis (PCA) was used for data exploration purposes and for identifying tendencies of the CH_4 yield based on the different levels of the pretreatment variables. The software used for this purpose was The

Table 1 Data matrix of ultimate CH_4 yields of digested manure fibers treated with AAS under different conditions used for PCA[a]

No. of experiment	Temperature of AAS (°C)	Duration of AAS (days)	NH_3 concentration of AAS (% w/w)	CH_4 yield (ml CH4/g TS)
1a	22	1	32	106.2243
1b	22	1	32	111.5652
1c	22	1	32	107.0878
2a	22	3	32	143.8944
2b	22	3	32	128.7069
2c	22	3	32	141.5351
3a	22	5	32	139.7697
3b	22	5	32	126.7412
3c	22	5	32	128.7438
4a	55	1	32	110.8356
4b	55	1	32	119.3204
4c	55	1	32	110.6114
5a	55	3	32	125.8958
5b	55	3	32	134.3595
5c	55	3	32	132.4131
6a	55	5	32	119.7610
6b	55	5	32	124.3537
6c	55	5	32	124.6628
7a	22	3	32	144.0650
7b	22	3	32	127.2090
7c	22	3	32	163.6780
8a	22	3	25	163.3950
8b	22	3	25	153.7280
8c	22	3	25	187.3500
9a	22	3	20	181.0820
9b	22	3	20	180.0060
9c	22	3	20	144.2920
10a	22	3	15	145.1680
10b	22	3	15	177.6820
10c	22	3	15	167.3930
11a	22	3	10	146.3340
11b	22	3	10	171.3740
11c	22	3	10	177.0590
12a	22	3	5	166.8230
12b	22	3	5	165.6650
12c	22	3	5	156.4660
13a	22	1	25	218.9580
13b	22	1	25	202.4960
13c	22	1	25	217.1620
14a	22	3	25	205.0210
14b	22	3	25	227.4380
14c	22	3	25	222.6920
15a	22	5	25	158.9800
15b	22	5	25	178.3310
15c	22	5	25	162.6690
16a	22	1	5	169.4050
16b	22	1	5	201.0910
16c	22	1	5	240.9190

Table 1 continued

No. of experiment	Temperature of AAS (°C)	Duration of AAS (days)	NH_3 concentration of AAS (% w/w)	CH_4 yield (ml CH4/g TS)
17a	22	3	5	197.9670
17b	22	3	5	191.9950
17c	22	3	5	209.1050
18a	22	5	5	205.2330
18b	22	5	5	185.5600
18c	22	5	5	196.9940

[a] Data graphically presented in Jurado et al. [17] and Mirtsou-Xanthopoulou et al. [9]

Unscrambler®X 10.3 (CAMO, Norway). Standardization of the data matrixes was performed by first subtracting the mean from the values and then dividing them by the standard deviation of the corresponding variable. This way, the variables that initially have a very different range of variance become more comparable as the variance becomes even.

Results and Discussion

During the last years, different pretreatments have been tested on manure fibers (both raw and digested), for increasing the biogas or methane yield of manure-based anaerobic digestion systems. Hartmann et al. [21] tested the mechanical maceration of manure and found a 25 % increase on biogas production while the resulted increase was insignificant when only raw manure fibers were treated. Raphique et al. [22] tested a thermal, a thermochemical and a chemical [with $Ca(OH)_2$)] pretreatment of raw manure fibers and reported a 28 %, a 72 % and a negative increase of CH_4 yield respectively. In another study, thermal steam explosion was tested on raw manure fibers and resulted in a 50 % increase of CH_4 yield [23]. While the highest increase of CH_4 yield achieved up to now from pretreated raw manure fibers is 178 % by AAS [17], pretreating digested manure fibers has proved to be more efficient. Angelidaki and Ahring [24] reported a 17 % increase of biogas potential from digested manure fibers treated by mechanical maceration, and a 30 % increase when treated with a biological treatment. Bruni et al. [25, 26] investigated the effects of a mechanical, a chemical, a thermal and an enzymatic pretreatment of digested manure fibers and found that the most significant CH_4 increase was generated by steam treatment with H_2SO_4 (67 % increase) and treatment with CaO (66 % increase). Finally, wet explosion of digested manure fibers resulted in a 136 % increase of CH_4 yield [27]. In comparison to the results obtained so far from these pretreatments, AAS of digested manure fibers has achieved an increase of CH_4 yield up to 205 % (265 % at 17 days of digestion) when compared to non-treated fibers [9]. In order to identify the important factors that affected the performance of AAS on digested manure fibers, a statistical tool was used for analyzing the results obtained up to now.

PCA is an exploratory data analysis method aiming at separating the important information hidden in one data matrix from the noise [28]. In PCA, a new orthogonal axis-system is defined in a way that each principal component (PC) represents an axis, along which, the maximum variation within the data is described. The first PC (PC1) is defined by the maximum variance direction (axis) of the data set; the second PC (PC2) is orthogonal to PC1 modeling the second maximum variance direction, and so on. The transformed data, scores, can be plotted in the new PC space (score plot). Interpretation of the variance modeled by each PC allows statements as to the reasons why the data are distributed as revealed in the score space, and is assessed by the loadings relationships (loadings plot), in which the contribution of the initial variables on the construction of PCs can be assessed. This way the hidden data structure, e.g. groupings, clusters, trends between objects (score relationships) and the correlation of variables responsible (loading relationships) is revealed.

PCA was conducted for exploring the effects of temperature, duration and NH_3 concentration of the AAS pretreatment of digested manure fibers on CH_4 yield as resulted from batch tests. The first data set concerns ultimate CH_4 yields obtained from BMP tests under different conditions of AAS as described earlier. In Fig. 2 the scores plot of the first data set is shown. The correspondence of the names of the samples to the conditions of the AAS pretreatment is given in Table 1. In Fig. 3 the loadings plot is presented where the correlation of the different variables can be viewed and from where assistance can be provided for the interpretation of the scores plot.

As revealed from the loadings plot (Fig. 3), the first PC explains 50 % of the total data variance and models three of the variables (CH_4 yield, temperature, and NH_3 concentration) while the second PC models an additional 25 % of the variance, expressing only one variable, the duration of the AAS. As observed in the loadings plot (Fig. 3), CH_4

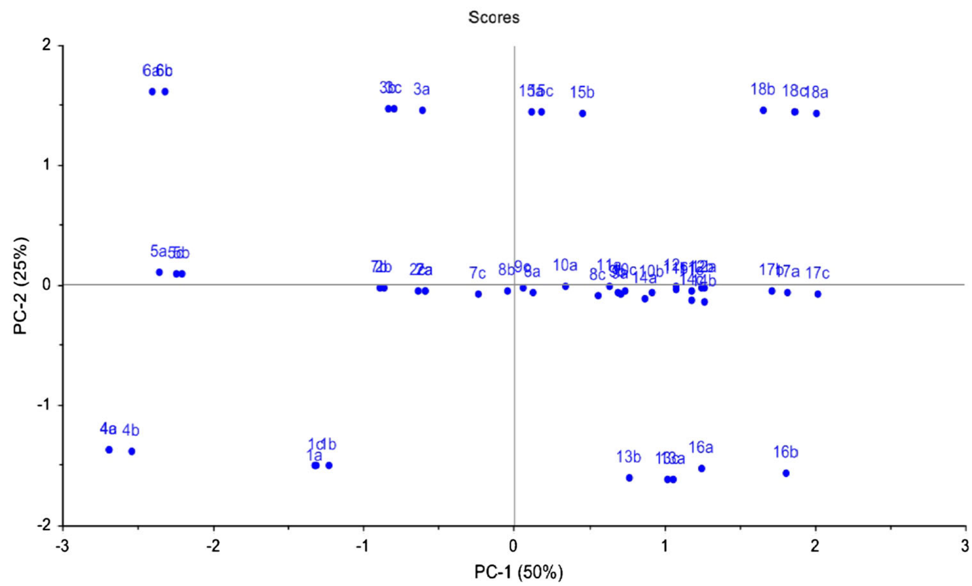

Fig. 2 Scores plot of the PCA of experiments with AAS pretreated digested manure fibers under different temperature, duration and NH_3 concentration of the reagent. The *plot* shows the correlation of the experiments in the PC1 versus PC2 space. The numbers of the experiments are explained in Table 1

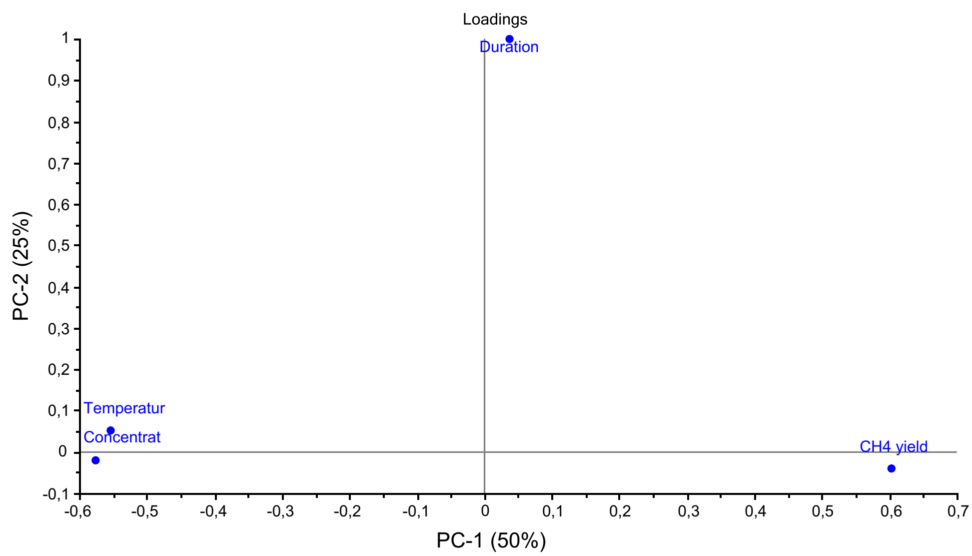

Fig. 3 Loadings plot of the PCA of experiments with AAS treated digested manure fibers. The loadings plot shows the correlation of the four variables (temperature, NH_3 concentration, duration and CH_4 yield) of the experiments in the PC1 versus PC2 space

yield seems to be strongly, negatively correlated to both temperature and NH_3 concentration. On the other hand, the duration of the pretreatment seems not to be correlated to the CH_4 yield, as duration varies along PC2 and not along PC1. This can also be concluded from the scores plot in Fig. 2. PC2 clearly separates the batches according to duration; along this PC (from negative to positive values) the duration increases from 1 to 5 days, forming three horizontal groupings. On the other hand PC1 separates samples according to temperature of the pretreatment, high temperature (55 °C) samples are situated on the extreme left, and according to NH_3 concentration, samples from the left to the right generally present a decreasing NH_3 concentration.

Based on the position of CH_4 yield on the loadings plot illustrated in Fig. 3, the highest CH_4 yields correspond to the samples on the right of the scores plot. These samples were all treated at room temperature (22 °C) and at all different durations (1, 3 and 5 days). Regarding the NH_3 concentration with which these samples were treated, although the general trend is that the concentration decreases along PC1, the different levels within the group of samples on the right of the plot are difficult to distinguish (Fig. 2). This might be due to the fact that not all different concentration levels were tested on all different temperature and duration combinations due to logistical constraints; this data set may therefore not have been optimal for describing all main as well as interacting effects between concentration and the rest of variables. On the contrary it is safe to conclude that the highest level of concentration has the lowest effect on increasing CH_4 yield as all samples treated with 32 % w/w NH_3 (aq.) are situated on the left of the scores plot. PCA of the second data set (CH_4 yield at ca. 18 days) has shown the same correlations (results not shown), which means that the influence of these factors on CH_4 yield at the ranges tested, does not change over the duration of digestion.

Few studies have been conducted assessing the performance of AAS on lignocellulosic biomasses under different conditions. Regarding the importance of NH_3 concentration used for the pretreatment, literature seems to be ambiguous. Li et al. [14] have tested AAS on wheat straw under 0–30.8 % w/v NH_3 (aq.) and found that the CH_4 yield increased when the NH_3 concentration increased up to 18 % but not when it was further increased. Song et al. [15, 16] reported that increasing the ammonia concentration from 1 to 4 % w/w and up to 10 % w/w led to an enhanced CH_4 yield from pretreated rice straw and corn straw respectively. On the other hand, Ko et al. [29] mentioned no significant effect on enzymatic digestibility of pretreated rice straw when increasing the NH_3 concentration from 12 to 28 % w/w, and actually reported a decrease of digestibility when the highest NH_3 concentration was applied. Other studies have shown a very slight increase of digestibility of the treated biomass or of CH_4 yield when the concentration was increased [12, 30, 31]. The importance of the NH_3 concentration of the pretreatment might be attributed to the different pH along the different concentrations of NH_3 (aq.) solutions, as these two factors are strongly correlated. According to this and to the conclusions derived from this PCA, the concentration of NH_3 used for the pretreatment seems to be an important though not a decisive factor on the success of AAS, except when concentration of NH_3 is very high resulting to a negative effect.

In agreement to this survey, Li et al. [14] found the temperature of the pretreatment to be very influencing on the CH_4 yield. They mention a positive correlation between these two variables, attributing this observation to a higher degree of delignification. In general, an increase of temperature has been linked to a higher lignin removal, which is often associated to a higher digestibility of lignocellulosic materials. A strong correlation between digestibility and lignin removal has been observed in more studies [29, 32]. Nonetheless, studies on compositional changes of manure fibers revealed that no apparent lignin removal had taken place after the AAS pretreatment [33]. The authors suggest that the increased CH_4 yield is probably a result of the increased exposure of cellulose, resulted from the swelling that AAS caused. This theory is in accordance with previous works stating that ammonia treatment causes a "fiber expansion" that facilitates the enzymatic hydrolysis of lignocellulosic biomasses [34]. Moreover, it has been reported that enhanced methane yield could result from very small changes on lignin matter rather than only when delignification takes place [14].

The different observations on the effect of ammonia on lignocellulosic structures might be attributed to the different solid-to-liquid (S:L) ratios used for the pretreatment. In the case of pretreated manure fibers the S:L ratio was ca.1:3, [1 g material: 2.8 ml NH_3 (aq.) if considering that the treatment was performed with 10 ml NH_3(aq.) per 1 g TS and the TS content of digested fibers was ca. 28 %] [9, 17]. This ratio is much higher than the ratios of 1:6 and 1:10 that are usually used for other biomasses [14, 32, 34]. The S:L ratio has been pointed out as a very influencing factor for NH_3 pretreatment of lignocellulosic biomasses [35] and previous studies have shown that a decrease of the S:L ratio causes an increase of delignification and of enzymatic digestibility. Yoo et al. [31] studied the effect of three different S:L ratios of AAS on barley straw (i.e. 1:3, 1:6 and 1:10) and found that a decrease of the S:L ratio resulted to higher delignification and higher enzymatic digestibility. Similar results were obtained on a study of AAS on corn stover at S:L ratios from 1:2 to 1:10 [30]. In conclusion, AAS at high S:L ratios may affect lignocellulosic biomasses mainly by a different mechanism (swelling) than removing the lignin and this might be the reason why increasing temperature did not cause an increase of the CH_4 yield from pretreated manure fibers. For a deeper understanding of the nature of the AAS pretreatment, it is essential to test this hypothesis in further investigations.

Finally, some studies have reported the duration of AAS to be important in terms of enzymatic digestibility of lignocellulosic biomasses. Kim and Lee [30] and Yoo et al. [31] tested different durations of AAS on corn stover and barley straw respectively and both groups concluded that increasing the duration had a positive effect only up to 12 h, and then remained stable. Although in both cases the rest of the variables were fixed at constant values, strong interaction effects exist between duration and the two variables (which agrees with Chandel et al. [36]), with the interaction of duration and concentration to be the most important. In all these studies, the effect of pretreatment duration was examined only at high temperatures (over 50 °C) and high NH_3 concentrations (15–28 %) and in contrast to the data set examined at the present study, the ranges tested included also lower durations (down to 6 h). This indicates that reducing the pretreatment duration to a few hours might increase considerably the importance of this factor.

Interactions between the AAS parameters have been mentioned in more studies, and given the nature of the variables it is well expected that correlations exist. Low temperature is expected to increase the importance of duration and vice versa [34]. Nevertheless, AAS on different lignocellulosic biomasses seems to act with a different mechanism and conclusions on linear interactions between the variables are probably precipitous. For instance, low temperature (up to 55 °C) of AAS on manure fibers did not result to a higher importance of the duration of the pretreatment as discussed earlier. This might be due to a parallel interaction with the NH_3 concentration of the pretreatment or more likely with the S:L ratio. Additionally, it has to be taken into account that the composition of manure fibers is considerably different from the composition of the different straws that have been tested up to know, mainly due to their much higher moisture content (usually around 70 %). Thus, it could be hypothesized that the duration of the pretreatment may become important if the test range widens up to higher values. However, very high durations might not be of interest for industrial applications as they are not economically favorable. The selection of the ranges within the variables that will be optimized should be chosen very carefully and after taking into consideration all possible limitations derived from a full scale application.

Given the uncertainty of the mechanism of AAS under the whole range of each variable, and the complexity of a multivariable system, a suitable experimental design is necessary in order to conclude on the possible interactions between the pretreatment variables affecting the CH_4 yield of pretreated manure fibers. There appear to be four most influential factors, i.e. temperature, NH_3 concentration, AAS duration and the S:L ratio. Instead of standard experimental design which requires a substantial total number of runs if all possible interactions shall be covered (three levels, four factors), an alternative design that will allow outlining the full impact of all potential interactions would be more appropriate, i.e. a so-called random design, as outlined in [28]. This design facilitates yield modeling using partial least squares regression based on a reduced number of runs, sacrificing formal ANOVA significance evaluation for direct interaction-and-main effects influences as revealed in pertinent loading-weights relationships. Based on this first foray, final optimization is based only on the most influential factors and the interactions revealed; an efficient two-stage optimization.

Conclusions

AAS has been recently proved to be a pretreatment of great potential for increasing CH_4 yield of manure fibers. The AAS pretreatment of manure fibers coupled with a successful NH_3 recovery step could lead to a more sustainable biogas production allowing biogas plants to operate solely on manure. Furthermore, integration of the digester effluent to the NH_3 recovery step could produce an excess of NH_3 to be used for the reduction of NO_x, providing this way a more environmental friendly operation of gas engines when the biogas produced is used for electricity generation.

In this study, the performance of AAS as a pretreatment of digested manure fibers under different conditions was explored in terms of maximum CH_4 yield, based on previous experiments. PCA revealed that within the ranges of pretreatments and combinations tested, temperature and NH_3 concentration have more influence on CH_4 yield, while the duration of AAS is less important. Optimization of AAS of manure fibers must be conducted for guaranteeing maximum performance, and the effect of lower values of NH_3 concentration and duration should be included in the investigation. The most important factors of AAS that seem to affect the performance of the pretreatment and should be optimized are temperature, NH_3 concentration, duration and S:L ratio. It will be necessary to establish an experimental design that will allow a full impact also from interactions between these four factors, i.e. a so-called random design.

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Paper II:

“Screening for the important variables of aqueous ammonia soaking as a pretreatment method for enhancing the methane production from swine manure fibers”

Screening for the important variables of aqueous ammonia soaking as a pretreatment method for enhancing the methane production from swine manure fibers

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Abstract
Manure-based anaerobic digestion often results to be an economically non-feasible process. During the last years many methods for the pretreatment of the solid fraction of manure (raw manure fibers) have been tested in order to increase the methane yield and make this process profitable. One of the most promising treatments is aqueous ammonia soaking (AAS). In previous tests, the methane yield of AAS-treated raw swine manure fibers increased by 178% as compared to the methane yield of non-treated manure fibers. Nevertheless, the efficiency of the pretreatment on increasing the methane yield depends on the specific conditions under which it is carried out. In the present study, a series of screening experiments took place in order to identify the most influencing AAS parameters affecting the methane yield of pretreated raw swine manure fibers. The solid-to-liquid ratio, the duration and the concentration of ammonia in the reagent were found to be the most decisive factors for the success of the pretreatment.

Keywords
Aqueous ammonia soaking; manure fibers; methane; biochemical methane potential; screening; partial least squares regression

INTRODUCTION

Livestock manure has traditionally been used as a substrate for biogas production as its treatment is essential for reducing the organic loading and nutrient loss that occurs when applied directly to the fields. Nevertheless, the anaerobic digestion process of manure often results to be dependent on the addition of extra organic materials for enhancing the biogas production. Alternatively, the pretreatment of the solid fraction of manure (manure fibers) that presents the largest methane potential, could make biogas production of solely manure a more economically feasible process. Aqueous ammonia soaking (AAS) of swine manure fibers seems to be a very promising solution. It is a very simple process with a low energy demand, during which ammonia can be relatively easily removed and recycled due to its high volatility, resulting thus in minimum consumption of chemicals. Moreover, previous experiments have shown that AAS of raw manure fibers (Jurado et al. 2013) as well as of the solid fraction of digested manure (digested manure fibers) (Mirtsou-Xanthopoulou et al. 2014) can significantly increase the methane production.

The maximum increase of methane yield obtained up to now from AAS of raw and digested manure fibers was 178% and 205% respectively, when compared to the methane yield of non-treated fibers (Jurado et al 2013, Mirtsou-Xanthopoulou et al 2014). Nevertheless, the effect of the pretreatment on the methane yield depends on the specific conditions under which AAS is performed. Parameters that have been found to be potentially important for an efficient AAS are the temperature and the duration of the pretreatment, the concentration of ammonia in the reagent, and the solid-to-liquid (S:L) ratio (Lymperatou et al 2014). In this study, a series of screening experiments was carried out for identifying the most important parameters of AAS affecting the methane yield of raw swine manure fibers. The effects of the different parameters were assessed based on Partial Least Square-Regression (PLS-R).

MATERIALS AND METHODS

Substrate and analytical methods

The raw manure fibers used for the experiments were provided from Limfjordens Bioenergi (Denmark) and were separated from raw swine manure by a decanter centrifuge. The fibers were stored at -20°C until used for the experiments. The total solid (TS) and volatile solid (VS) content of the raw manure fibers were 34% and 23% respectively. The inoculum used for the BMP tests originated from an industrial-scale mesophilic anaerobic digester (Hashøj Biogas, Denmark) that treats swine manure, bovine manure and other organic wastes. After the AAS treatment, the NH_3 was removed from the treatment mixture by vacuum distillation. NH_4^+-N determination was carried out by Hach Lange kit LCK-305. The CH_4 production was monitored with a gas chromatograph equipped with a thermal conductivity detector (GC Mikrolab GC82-22).

Experimental set up

Aqueous ammonia soaking treatment. The importance of four parameters of AAS were tested, namely temperature (T), duration of AAS (D), concentration of NH_3 in the reagent (A) and the solid-to-liquid ratio (SL) at which the pretreatment took place. The different levels of the parameters tested are shown in Table 1. In total, sixteen pretreatments of raw manure fibers were performed based on a random design as described in Esbensen (2001). The manure fibers were placed in 2 l vials and the respective solution of NH_3 (aq.) was added. The vials were sealed and left intact until the duration of the pretreatment had finalized. Subsequently, the NH_3 was removed to an NH_4^+-N concentration lower than its level in the inoculum, ensuring thus no inhibition of the methanogenic phase would occur.

BMP tests. The pretreated raw manure fibers were placed in 320 ml vials together with inoculum under anaerobic conditions at a ratio of 40 ml inoculum/g TS fibers added. BMP tests with inoculum and non-treated raw manure fibers, and BMP tests with only inoculum served as controls and blanks respectively. All BMPs were set at triplicates. The vials were incubated at 37°C and the CH_4 production was monitored twice per week during 48 days of digestion.

Table 1. Levels of AAS parameters tested in the screening experiments.

Level	Temperature (T) in °C	Duration (D) in h	Solid-to-Liquid ratio (SL) in g fibers/ ml reagent	Ammonia concentration (A) in % w/w
1	20	12	1:10	0,6
2	35	48	1:6	15
3	50	120	1:3	32

Partial Least Squares Regression (PLS-R)

The identification of the important variables was assessed by PLS-R, performed with the software The Unscrambler (CAMO, Norway). A data matrix was formed based on the 16 experiments, with 5 columns corresponding to the four AAS parameters, i.e. temperature, duration, NH_3 concentration and solid-to-liquid ratio, (considered as X variables), and the resulting average cumulative CH_4 yield (Y variable) of each set of triplicates after 16 days of digestion.

RESULTS AND DISCUSSION

CH_4 yields of AAS-treated raw manure fibers

The obtained CH_4 yields of the raw manure fibers (treated under different conditions of AAS) and of non-treated fibers (NP) are presented in Figure 1. The experiments lasted 48 days, after which the cumulative CH_4 yield in most vials was stabilized. As derived from figure 1, the increase of the cumulative CH_4 yield of AAS-treated fibers at 48 days of digestion ranged from 46,92% (experiment 4) to 196,08% (experiment 6) in comparison to the cumulative CH_4 yield of non-pretreated (NP) fibers. It appears that the ranges of the AAS variables tested produced the sufficient variation in the response (CH_4), thus justifying the necessity of screening the AAS treatment conditions for enhancing the CH_4 yield of raw manure fibers. Apart from the cumulative CH_4 yield after 48 days of digestion, the rate of CH_4 production seems to have been affected in some cases. Experiments 14 and 15 clearly showed an increased rate of CH_4 production during the first 12 days of digestion, whereas thereafter the production rate was reduced.

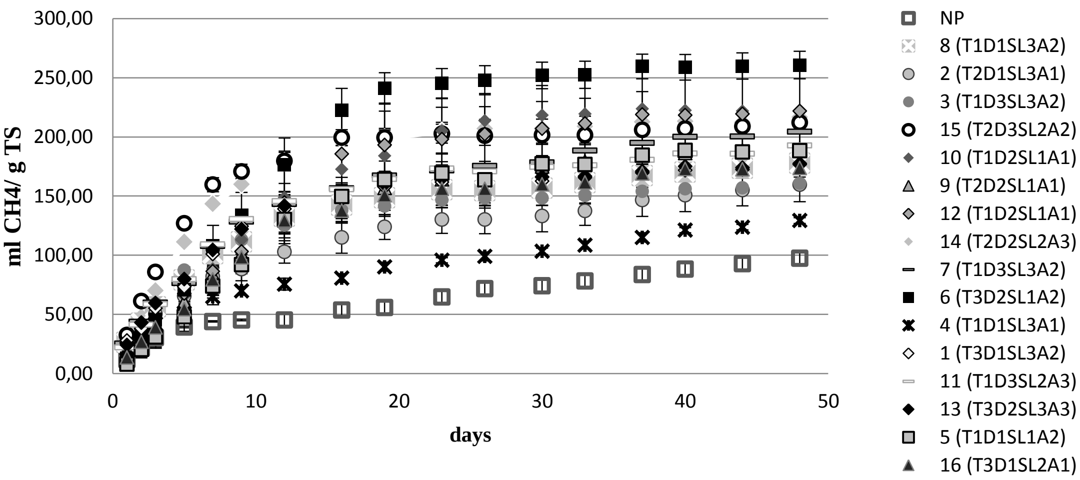

Figure 1. Cumulative CH_4 yield of raw swine manure fibers treated with AAS under different conditions. Each number corresponds to an AAS treatment, the conditions of which are given in brackets next to the number. T, D , A and SL stand for temperature, duration, ammonia concentration and solid-to-liquid ratio respectively and numbers to the levels of each parameter as shown in Table 1. NP stands for non-pretreated fibers.

The four parameters (T, D, A and SL) of AAS chosen as potentially important appear to have affected differently the CH_4 yield of the treated manure fibers. The highest CH_4 yields obtained after 16 days of digestion correspond to the pretreatments 6, 14, 15, 10 and 12 (Fig.1). The temperature during these pretreatments was 35°C for the two first, 50°C for the third and 20°C for the two last, indicating that the temperature was not the reason for which these experiments produced more CH_4. The same applies with the NH_3 concentration in the reagent; that is pretreatments 6, 14, 15, 10 and 12 were performed at different concentrations of NH_3. On the other hand the solid-to-liquid ratio at which these experiments were performed were all at the medium or lowest level, while the pretreatments performed at the highest solid-to-liquid ratio performed rather poorly, e.g. experiments 4, 2 and 3. Thus, it appears that this parameter, the solid-to-liquid ratio, plays a decisive role in the AAS of raw manure fibers for increasing their CH_4 yield. Finally, the duration of the pretreatment seems to have affected the performance of the experiments, as most treatments performed at low duration produced less CH_4 (Fig.1).

Besides the general trends that can be easily seen from figure 1, it is difficult to conclude on the reasons why some of the AAS-treated fibers produced more CH_4. This is probably due to interactions between the four parameters that are difficult to recognize from Fig.1. Therefore a statistical tool, PLS-R, was used for identifying the most important AAS parameters affecting the CH_4 yield of AAS-treated raw manure fibers.

Important AAS variables affecting CH_4 yield – PLS-R results

In Figure 2 the two plots, Scores and Loadings, as resulted from the PLS-R analysis are presented. Two factors were considered to be optimal for the analysis, as the subsequent factors did not add significantly more information. The two-factor PLS-R explained 82% of the variance of the cumulative CH_4 yield and 56% of the variance of the AAS parameters. The fit of the experimental data to the model was satisfactory (R^2 = 0.82) and the Root Mean Square Error was 15.09. In the Scores plot (left on Fig.2) the average of the BMP triplicates 1-16 are plotted in the Factor 1-Factor 2 space. From this plot, it may be seen that the experiments are generally spread and form three groupings. The left group of experiments corresponds to the ones with the lowest CH_4 yield; the right group corresponds to the experiments with the highest CH_4 yield, while the rest of the experiments which produced middle-value CH_4 yields are situated in the middle. This can also been seen from the loadings plot (right on Fig.2); the CH_4 yield is well expressed from Factor 1, along which from negative to positive values the CH_4 yield increases. From this plot, it is also possible to evaluate the correlation between CH_4 and the four AAS parameters. According to this, the CH_4 yield is strongly and negatively correlated to the solid-to-liquid ratio, confirming the observations from Fig.1. On the other hand the temperature appears to be positively correlated to the CH_4 yield, although not that importantly. Finally the duration and the NH_3 concentration of the AAS treatment seem to affect the CH_4 yield of the treated fibers in a positive way, though not that strongly as the solid-to-liquid ratio.

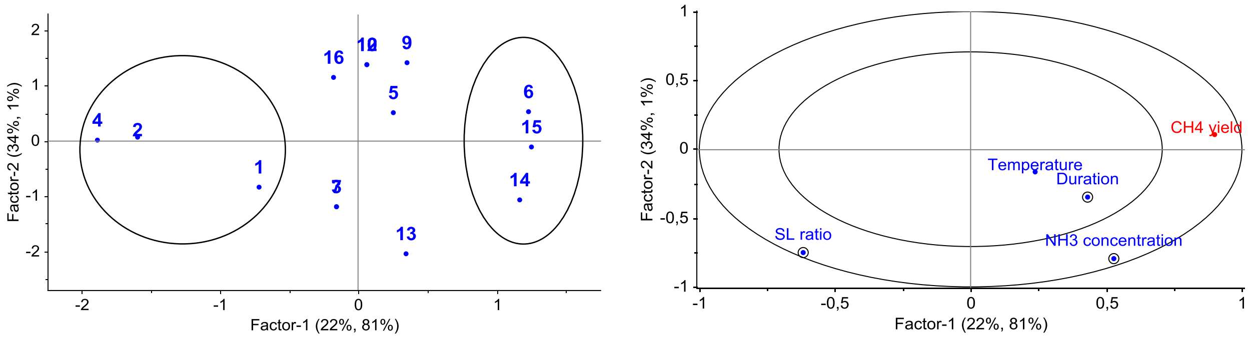

Figure 2. Scores (left) and Loadings plot (right) of PLS-R of the cumulative CH_4 yields after 16 days of digestion of raw manure fibers treated with AAS under different temperature, duration, ammonia concentration in the reagent and solid-to-liquid ratio (SL). Factors of AAS in circle correspond to the ones found by PLS-R to have an important effect on the CH_4 yield. The numbers shown in the Scores plot correspond to the number of the AAS pretreatment, the conditions of which can be seen in Fig.1.

In conclusion, the factors that appear to be more strongly correlated with the CH_4 yield of AAS-treated raw manure fibers are the solid-to-liquid ratio, the duration and the NH_3 concentration at which the AAS is performed. The solid-to-liquid ratio is negatively correlated to the CH_4 yield which means that larger liquid volumes are more likely to give a higher CH_4 yield. In an industrial application, this would be translated to a larger pretreatment reactor. The duration and NH_3 concentration of the AAS are positively correlated to the CH_4 yield of the treated fibers. This can provide some flexibility to the configuration of the process. In order though to develop a full view of how sensitive the CH_4 yield of AAS-treated raw manure fibers is to variations of the AAS parameters, optimization of the three most influencing factors, solid-to-liquid ratio, duration and NH_3 concentration is needed.

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APHA, American Public Health Association/American Water Works Association/Water Pollution Control Federation 2005 Standard methods for the examination of water and wastewater 21st ed. Washington DC, USA

Paper III:

“Optimization of Aqueous Ammonia Soaking of manure fibers by Response Surface Methodology for unlocking the methane potential of swine manure”

"Optimization of Aqueous Ammonia Soaking of manure fibers by Response Surface Methodology for unlocking the methane potential of swine manure"

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ABSTRACT

Swine manure mono-digestion often results to economically non-feasible processes, due to the high dilution and ammonia concentration together with the low degradation rates it presents. The effects of different parameters of Aqueous Ammonia Soaking (AAS) as a pretreatment for improving the digestion of manure fibers when coupled to an ammonia removal step were investigated in this study. Response Surface Methodology was followed and the influence and interactions of the following AAS parameters were studied: NH_3 concentration, duration and solid-to-liquid ratio. The mild conditions found to be optimal (7% w/w NH_3, 96 hours, and 0.16 kg/l) in combination to a significant increase of the short term CH_4 yield (244% in 17 days), make this pretreatment a promising solution for improving swine manure mono-digestion. Furthermore, compositional analysis of the manure fibers revealed significant solubilization of hemicellulose, while no lignin removal or loss of cellulose occurred under optimal conditions.

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Keywords: anaerobic digestion, aqueous ammonia soaking, response surface methodology, manure, pretreatment

1. Introduction

The interest in developing efficient renewable energy production processes grows continuously as a response to the future limited availability of fossil fuel resources and to the greenhouse effect. Anaerobic digestion constitutes one of the oldest and most established renewable energy production processes, both in developed and developing countries (Bond and Templeton, 2011). The fact that the biogas produced can be used for direct heating, electricity production, to replace natural gas or as a vehicle fuel (when upgraded), ensures that anaerobic digestion processes will represent a major role in the future energy production sector (Holm-Nielsen et al., 2009).

Livestock manure is one of the most extensively used substrates for anaerobic digestion due to the worldwide expansion of the livestock production sector and to its rich content in nutrients and microorganisms, that lead to the spontaneous production of biogas under anaerobic conditions. However, due to the low price of biogas, the low conversion rate of manure to biogas and the high water content attributed to current management practices in farms, the anaerobic digestion process of solely manure often results to be economically non-feasible (Møller et al., 2007). This fact has led to the concept of co-digestion where manure is enriched with diverse organic materials that present a higher biogas production rate, such as whole-grain crops and residues from crops or from the food industry (Asam et al., 2011). Undoubtedly this practice offers some benefits to the operation of a biogas plant, i.e. improves the characteristics of the input material by facilitating the adjustment of the C:N ratio and the dry matter content. However, it renders biogas plants dependent on the availability of these extra materials that might be scarce in comparison to manure.

For instance, the amount of livestock manure treated anaerobically in Denmark does not exceed 8% of the total annual production, whereas common substrates for co-digestion are of limited availability (Mikkelsen et al., 2016). This fact in turn, increases the potential pollution of the atmosphere by greenhouse gas and ammonia emissions due to other manure management practices (Chadwick et al., 2011). Therefore, it is important to develop technologies that will improve the methane efficiency when manure is the sole substrate and thus they will lead to an increase of the amount of manure treated and of the energy recovered.

The challenge of improving the conversion of manure to biogas is mainly due to the refractory nature of the lignocellulosic content as well as to the high ammonia content that often characterizes it (Sawatdeenarunat et al., 2015). Usually only 30-50% of the organic matter of manure is degraded during anaerobic digestion in biogas plants (Christensen et al., 2007). Aiming at overcoming these limitations, various researchers have tested different pretreatments, e.g. mechanical, thermal and chemical treatments (Angelidaki and Ahring, 2000; Bonmati et al., 2001; Bonmatí and Flotats, 2003; Carrère et al., 2009; González-Fernández et al., 2008), and in some cases a significant increase of digestibility in terms of biogas production or methane yield has been observed. Nevertheless, only the solid fraction of manure (manure fibers) could be targeted as it presents the concentrated recalcitrant fraction with the highest theoretical methane potential (Angelidaki and Ahring, 2000) as well as the majority of organic N. This way, the volume of material to be pretreated is significantly reduced which results to be economically more attractive for large scale applications. A separation of animal slurry to a solid and liquid fraction, which is possible by various available technologies (Hjørth et al., 2011; Møller et al., 2000), reduces transportation costs (Asam et al., 2011) and provides more flexibility on increasing the dry matter content of the influent addressing this way the high dilution of manure (usually less than 8% dry matter content while typical anaerobic digesters can operate with an influent of up to 12% dry matter) (Frandsen et al., 2011).

Similarly to other lignocellulosic biomasses, many pretreatments have been suggested for improving the degradability of manure fibers. Chemical pretreatments, while often highly efficient, are considered more difficult to implement in large scale due to the extra costs of chemical consumption. Among them, Aqueous Ammonia Soaking (AAS) has been identified as a promising pretreatment for lignocellulosic biomasses since ammonia is the only chemical used and is expected to be relatively easy to recover due to its high volatility. Especially in the case of swine manure digestion, an ammonia removal step could also partly alleviate the process from high ammonia loadings. On this line, some biogas plants are already equipped with ammonia stripping installations, facilitating thus an implementation of the pretreatment (Frandsen et al., 2011). Moreover AAS can take place at low temperatures and ambient pressure, reducing thus the energy input requirements. AAS has been tested so far on various biomasses under different configurations mainly for increasing ethanol production and sugar release (Kim et al., 2016, 2008; Ko et al., 2009; Yoo et al., 2013) but also for enhancing methane production (Antonopoulou et al., 2015; Hashimoto, 1986; Jurado et al., 2013a, 2013c; Li et al., 2015; Mirtsou-Xanthopoulou et al., 2014). Generally, AAS is considered to improve the hydrolysis of lignocellulosic biomasses by acting selectively on lignin while preserving carbohydrates (Carrère et al., 2016; Kim et al., 2016), facilitating thus the access of enzymes to carbohydrates. Besides the promising results of AAS applied to different feedstocks for anaerobic digestion, it has been optimized so far only for wheat straw at elevated temperature (Li et al., 2015). Screening experiments on the effects of AAS under different conditions on the methane yield of manure fibers, showed that temperature was the least influencing factor, permitting thus a low energy input of the process (Lymperatou et al., 2015a). A comprehensive study on how the efficiency of the pretreatment is affected by the most influencing parameters (ammonia concentration, duration, and solid-to-liquid ratio) is essential prior to scaling up, as it facilitates the process design and the evaluation of the techno-economic feasibility, elucidating the actual potential of a pretreatment.

In the present study, AAS was applied on swine manure fibers in order to evaluate the efficiency of the pretreatment on increasing the methane yield under different conditions. For this purpose, Response Surface Methodology (RSM) was followed and the optimal conditions for maximizing the methane yield of pretreated manure fibers were determined. Furthermore, the solubilization of the biomass under different conditions was assessed and practical limitations are discussed. Finally, empirical models, able to predict the methane yield of the AAS-treated fibers as a function of the pretreatment conditions were developed.

2. Materials and methods

2.1 Substrate and Inoculum

The substrate used for the experiments was collected at the biogas plant Limfjordens Bioenergi (Mors, Denmark) that received manure fibers separated from raw swine manure by means of a mobile decanter centrifuge. Once collected they were sealed in plastic bags and stored at -20 °C until used. The content of the manure fibers in total solids (TS) was 35.13 ± 1.76% of wet mass, and in Volatile Solids (VS) 23.59 ± 0.84% of wet mass. The total Chemical Oxygen Demand (COD) of the manure fibers was 1.20 ± 0.01 g O_2/g VS. The inoculum used for the Biochemical Methane Potential (BMP) tests originated from a centralized full-scale mesophilic biogas plant operating on livestock manure and organic waste (Hashøj Biogas, Denmark). The inoculum was incubated at 37 °C for 9 days prior to use, for minimizing the endogenous biogas production. The main characteristics of the inoculum were 5.3 % TS, 3.7 % VS, 3.1 g NH_4^+-N /l, 6.73 g soluble COD/l, and pH 8.05.

2.2 Optimization Designs – Response Surface Methodology

The performance of the AAS pretreatment of manure fibers was tested under different conditions in order to find the optimal values of the parameters that were found to be the most influencing on the resulting CH_4 yield, based on screening experiments (Lymperatou et al., 2015a). These were the NH_3 concentration in the reagent, the duration of AAS and the solid-to-liquid (S:L) ratio. All AAS pretreatments were conducted at room temperature (20°C). Initially, a circumscribed Central Composite Design (cCCD) was followed, with the 3 independent variables varying at 5 levels: 0.9, 7, 16, 25, and 31.1% w/w NH_3 concentration, 4.8, 28, 62, 96 and 119.2 hours of duration, and 0.12, 0.16, 0.18, 0.22 and 0.32 kg fibers/ l reagent for the S:L ratio. In total, nineteen AAS pretreatments of manure fibers were performed, comprising of 8 cube points (2^3), 6 axial points where 1 variable was set to the maximum or minimum value while the rest of them were set at the middle values, and the central point (all variables set at the middle value) was replicated 5 times for allowing of estimation of the experimental error. Additionally, given that the VS determination is an indirect measurement, it was decided to model the CH_4 yield per g TS instead, in order to reduce the associated errors. A similar approach has been followed in more studies where CH_4 yield of lignocellulosic substrates is modelled (Monlau et al., 2012). The responses of the system were the cumulative CH_4 yield after 17 days of digestion ($CH_4\ 17d$) and the corresponding increase of CH_4 yield as compared to the non-pretreated (NP) fibers ($CH_4\ yield\ increase$), expressed in ml/g TS and % respectively, as resulted from biochemical methane potential (BMP) tests. Values of the volume of CH_4

yield reported are given at 20°C and 1 atm, unless otherwise stated. Additionally, the soluble COD ($solCOD$) was set as a response in order to evaluate which AAS parameters mostly affected the solubilization of manure fibers. The $CH_4\ yield\ increase$ achieved by the AAS-treated manure fibers was calculated as:

$$CH_4\ yield\ increase = \frac{CH_4\ yield_{AAS} - CH_4\ yield_{NP}}{CH_4\ yield_{NP}} * 100\ ,\ (eq.1)$$

Where:

$CH_4 yield_{AAS}$, the average of triplicates of the CH_4 yield of AAS-treated fibers under each set of conditions expressed in ml CH_4/ g TS

$CH_4 yield_{NP}$, the average of triplicates of the CH_4 yield of the NP fibers expressed in ml CH_4/ g TS

The first set of optimization experiments was followed by a second experimental design using a faced Central Composite Design (fCCD), where the independent variables were the NH_3 concentration and the duration of AAS. The ranges of the independent parameters studied in the fCCD were 1, 4, and 7% w/w NH_3 concentration and 96, 120 and 144 hours of duration.

The experimental results obtained from both designs (cCCD, fCCD) were analyzed by using Response Surface Methodology (RSM) with the statistical software Design Expert 9 (StatEase, USA). RSM is a statistical tool for studying the effects of independent parameters on one or more responses (dependent parameters), and permits the construction of an empirical model with the form:

$$Y = b + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

(*eq.2*)

Where Y is the dependent parameter (response); x_1, x_2, x_3 are the independent parameters NH_3 concentration, duration of AAS and S:L ratio respectively; $b\ is$ the intercept coefficient; b_1, b_2, b_3 are the regression coefficients expressing the main effect of each parameter on the response; b_{12}, b_{13}, b_{23} are the regression coefficients for the interaction effect of 2 independent parameters on the response; $b_{11,}, b_{22}, b_{33}$ are the regression coefficients for the quadratic effect of each independent parameter on the response. The regression coefficients are calculated by regression analysis of the experimental data. The results obtained are assessed by ANOVA. All terms expressing main effects, while only interaction and quadratic effects found statistically significant ($p < 0.05$) were included in the models. The quality of the models was assessed by the fit of the experimental data to the model (R^2), the closeness of R^2 to the adjusted R^2 (indicating that the terms included are sufficient for modelling the response), and the lack-of-fit test being insignificant ($p>0.05$). Furthermore, validation experiments were run for ensuring the CH_4 yield predictions of models were satisfactory. Based on the empirical models obtained, Response Surface graphs are constructed where the predicted response is plotted as a function of two independent interacting parameters in a three-dimension graph.

2.3 Aqueous Ammonia Soaking (AAS) pretreatment

The corresponding amount of swine manure fibers was placed in 2 l screw-capped laboratory bottles with 600 ml of the solution of aqueous NH_3 of the corresponding concentration and sealed for avoiding losses of the NH_3. The mixture was left intact until the end of the pretreatment. Once the pretreatment was finalized, an equal-to-reagent volume of tap water was added to the mixture for facilitating the vacuum evaporation of NH_3. A rotary evaporator (Buchi Rotavapor, Switzerland) was used for this purpose and all batches were evaporated until reaching a concentration of less than 1 g NH_4^+/l. This way it was ensured that no inhibition of the anaerobic microorganisms would occur, since the NH_4^+.-N level of the inoculum was higher than the level of the substrate. The evaporation was performed at 130 mbar with initial temperature of the evaporator's water bath set at 20 °C and progressively raised up to 40 °C, 50 °C, 60 °C, 80 °C with a total duration of 80 min. The initial solution of aqueous NH_3 used for the AAS pretreatment was of 32% w/w purity (Merck KGaA, Germany).

2.4 Biochemical Methane Potential (BMP) tests

The BMP tests were set in 320 ml infusion bottles with 1.5 g TS of manure fibers and 60 ml of inoculum. In each series of experiments, two additional BMP tests were set up, one with inoculum and NP manure fibers, used as control, and one only with inoculum used as blank. All BMPs were set in triplicate. Inoculum was added and the bottles were flushed with a mixture of 80% N_2/20% CO_2 for ensuring anaerobic conditions. The bottles were sealed with rubber stoppers, secured with aluminum crimps and placed in an incubator at 37 °C. The CH_4 production was monitored periodically until the end of the experiments. The CH_4 production of the BMP tests of both pretreated and NP fibers were corrected for the residual production of the inoculum by subtracting the CH_4 production of the blank tests. Preliminary BMP experiments of AAS-treated fibers showed similar yields when varying the organic loading from 0.3-3.0 g TS/60ml of inoculum, ensuring thus that no inhibition would occur due to the organic loading. The same behavior was observed from BMP tests of NP manure fibers at different organic loadings (data not shown).

2.5 Analytical methods

Determination of TS, VS and ash was carried out according to Standard Methods (APHA, 2005). NH_4^+-N and soluble COD determination was performed after the NH_3 evaporation step by Hach Lange kit LCK 305 and LCK 514 (Hach Lange ApS, Denmark) respectively; samples of the pretreated fibers were centrifuged at 10,000 rpm for 10 min and filtered through 0.45 μm. For the total COD determination, manure fibers were dried at 105 °C, milled to powder with a commercial coffee grinder and diluted with Millipore-grade water. Measurement was done by Hach Lange kit LCK914 (Hach Lange ApS, Denmark). Determination of biogas content in CH_4 was carried out by Gas Chromatography (GC82-22, Mikrolab Aarhus, Denmark). The GC was equipped with a Porapak Q packed column (6 ft. and I.D. 3 mm), coupled with a Thermal Conductivity Detector (TCD) and N_2 was used as a carrier gas.

Compositional analysis of NP and AAS-treated fibers was performed based on NREL's protocols. Specifically, samples followed a two-step extraction based on Sluiter et al., (2008); the first extraction step was performed with Millipore-grade water during 6 hours, followed by an ethanol (96% v/v) extraction for 24 hours using a Soxhlet apparatus (EV6 ALL/16 No. 10-0012, Gerhardt, Germany). Structural sugars, acid-insoluble lignin and acid-soluble lignin of the extracted samples were determined by following NREL's protocol (Sluiter et al., 2011). In order to determine the soluble components of the biomasses, NP manure fibers were diluted in Millipore-grade water, while for the pretreated fibers the liquid fraction was used. Oligosaccharides were determined after a dilute acid hydrolysis of the samples as described in Bjerre et al. (1996). For determination of free sugars, samples were acidified with H_2SO_4 0.1 M, centrifuged at 10,000 rpm for 10 min and filtered through a 0.22 μm filters. Carbohydrates and acetic acid detection and quantification was performed by HPLC (Shimadzu, USA) equipped with a refractive index and an Aminex HPX-87H column (BioRad) at 63 °C. A solution of 12 mM H_2SO_4 was used as an eluent at a flow rate of 0.6 ml/min. Acid-soluble lignin was also measured from the dilute acid hydrolysates. Elemental Analysis (EA3000, EuroVector Instruments & Software, Italy) of NP fibers and AAS-treated fibers was performed on both as-received and extractives-free basis samples in order to determine the insoluble and soluble N content. Acetanilide was used as a standard. For determination of organic N, the values obtained from Elemental analysis were corrected for the inorganic N content of each biomass; only the NH_4^+-N content of each biomass was taken into consideration for the corrections, and the amount of N in form of nitrates and nitrites was assumed to be negligible. The soluble organic N was calculated as the difference between N content of as-received biomasses and N content of extractives-free biomasses.

3. Results and Discussion

3.1 Effect of AAS parameters on methane yield of swine manure fibers – Optimization (cCCD)

The cumulative CH_4 yields as resulted from the BMP tests of the first optimization step (cCCD) are presented in *Fig.1*. The control experiments (NP fibers) lasted in total 94 days, and the ultimate CH_4 yield observed was 182.56 ml/g TS, which is similar to the values reported by Møller et al., (2004) for swine manure fibers obtained from centrifugation. AAS affected positively the production rate under all conditions tested, though in a different degree. Here, it has to be mentioned, that in further experiments where NP manure fibers were soaked in water and then subjected to the same evaporation process as AAS-treated fibers, showed no effects on the resulted CH_4 yield due to the evaporation step (data not shown), thus any changes in the subsequent anaerobic digestion could be attributed to the AAS pretreatment. As it can be observed in *Fig.1*, the BMP tests of AAS-treated fibers reached the CH_4 yield of 38 days of the NP fibers in only 6-10 days depending on the conditions applied. The ultimate CH_4 yield of the AAS-treated fibers varied significantly based on the different conditions. While generally the same or higher ultimate yield as compared to the NP fibers was observed (*Fig.1*), a lower ultimate CH_4 yield was found from the batches pretreated with the lowest NH_3 concentration (experiment 9) or the lowest duration of AAS (experiment 11). Nevertheless, after 17 days of digestion the cumulative yields of the AAS-treated fibers were very close (> 75%) to their ultimate CH_4 yields (38 days). The cumulative CH_4 yields after 17 days of digestion were chosen for modelling, as the short-term yield could be a better indicator of a continuous process in comparison to the ultimate CH_4 yields for estimating the effects of the pretreatment within a reasonable duration of digestion. However, the effect of the AAS parameters on the ultimate CH_4 yields was also assessed in order to evaluate how the final biodegradability of the biomass was affected.

Two models were constructed based on the experimental results of the cCCD, namely *eq.3* and *eq.4*. The models constructed were highly significant ($p = 0.0001$) and the test of lack-of-fit was satisfactory ($p = 0.4177$). The effects that were found to be statistically significant to the responses ($p < 0.0500$) were the duration of AAS (x_2, $p<0.0001$), the interaction effect of the duration and the NH_3 concentration ($x_1 * x_2$, $p=0.0020$), and the quadratic effect of the S:L ratio (x_3^2, $p=0.0086$). The R^2 (0.84) was in good agreement with the adjusted R^2 (0.77), indicating that the effects included in the models are sufficient for modelling the responses. The final models for predicting the CH_4 yield of AAS-treated manure fibers after 17 days of digestion are:

$$CH_4\ 17d = 195.499 + 3.774 * x_1 + 1.328 * x_2 - 1176.160 * x_3 - 0.050 * x_1 * x_2 + 2540.255 * {x_3}^2, \ (eq.3)$$

$$CH_4\ yield\ increase = 213.21 + 5.94 * x_1 + 2.10 * x_2 - 1882.07 * x_3 - 0.08 * x_1 * x_2 + 4056.78 * x_3^2, \ (eq.4)$$

Where $CH_4\ 17d$ and $CH_4\ yield\ increase$, are expressed in ml/g TS and % as compared to NP fibers, respectively. All predictive models presented in this study, can be used for estimation of the CH_4 yield within the ranges of the experimental region, i.e. *eq.3* and *4* should be used for x_1, x_2 and x_3 values within the ranges [7, 25], [28, 96] and [0.16, 0.28] respectively.

The Response Surface graph of *eq.3* is presented in *Fig.2a*, where the predicted CH_4 yield is plotted as a function of the NH_3 concentration of the reagent and of the duration of AAS. The S:L ratio was set to a constant value of 0.16 kg/l as it was found not to interact significantly with the rest of parameters and corresponded to the optimum value. The duration of AAS appears to have had the strongest effect on the cumulative CH_4 yield, which is also depicted in the Response Surface graph, as the CH_4 yield rapidly increases along the axis of duration. On the other hand, the NH_3 concentration appears to have been important when the duration decreased down to 28 hours of pretreatment. Lastly, the S:L ratio resulted in high CH_4 yield when set at the maximum or minimum level of the experimental range. This is expressed in the model by the quadratic term of this parameter (x_3^2). According to *Fig.2a*, the optimal conditions of the AAS pretreatment of manure fibers for maximum CH_4 yield corresponded to 7% w/w NH_3 (aq.) and 96 hours of duration of AAS and at a S:L ratio of 0.16 kg fibers/l reagent. The prediction of *eq.3* for the CH_4 yield produced under these conditions, corresponds to 192.86 ± 11.14 ml/g TS, and based on *eq.4* to a 206.60% ± 17.68 increase of CH_4 yield as compared to NP manure fibers. Finally, it was verified by RSM that the ultimate CH_4 yields (after 38 days of digestion) presented the same trends (results not shown).

AAS has been optimized in the past for other lignocellulosic biomasses mainly for sugar release and ethanol production. For instance, AAS of barley straw was optimized for maximizing sugar recovery and the optimal conditions were found to be 15% w/w NH_3, 77.6°C, 12.1 hours and 1:8 S:L ratio (Yoo et al., 2013). In the case of rice straw, the optimal conditions were 21% w/w NH_3, 69°C and 10 hours of AAS for maximizing enzymatic digestibility (Ko et al., 2009). Optimal conditions of AAS reported for oil palm empty fruit brunches (Jung et al., 2011) and for oil palm fronds (Jung et al., 2012) corresponded to 21% w/w NH_3, 60°C, 12 hours and 7% w/w NH_3, 80°C, 20 hours, 1:12 S.L ratio respectively for maximizing ethanol production. Generally, the optimal conditions of the pretreatment appear to depend highly on the biomass studied, though a comparison of optimal conditions for different bioconversion processes is not that straightforward. For instance, in ethanol fermentation, hemicellulose is not consumed by wild-type yeasts, while in anaerobic digestion processes it is converted into CH_4 (Barakat et al., 2012). Consequently, optimal conditions of AAS of the same biomass but for different desired products may differ. As commented in Section 1, ammonia pretreatment has been optimized in the past for biogas production only for wheat straw (Li et al., 2015) and the optimal conditions reported were 14.8% w/w NH_3, 51°C and 27 hours of pretreatment, leading to a 56% increase of biogas yield. While harsher conditions appear to be necessary for pretreating wheat straw in comparison to manure fibers, direct conclusions could be misleading as the parameters chosen to be optimized and their ranges differ among the two studies.

3.2 Effect of AAS parameters on solubilization of COD

The AAS pretreatment resulted in significant solubilization of the swine manure fibers. As shown in *Table 1*, the NP fibers presented a soluble COD of 0.12% of total COD, while for the pretreated fibers soluble COD varied between 7.25% and 14.62% of total COD depending on the conditions of AAS. The factors that affected the solubilization of COD were mainly the duration of AAS ($p<0.0001$) and to a less extent the NH_3 concentration ($p=0.0275$). On the other hand, the S:L ratio appeared not to affect the solubilization of manure fibers ($p>0.05$). An increase of the two influencing factors produced an increase of the soluble COD measured. These findings are in line with previous studies where harsher conditions of AAS reduced the recovery of solids of pretreated rice straw (Ko et al., 2009). The model constructed was highly significant (p<0.0001), and the test of lack-of-fit was satisfactory ($p=0.1587$). The fit of the experimental data, while somewhat low ($R^2=0.77$), was in good agreement with the adjusted R^2 (0.74) indicating that the effects excluded from the model (S:L ratio and interaction and quadratic effects) did not provide significant information. The final model was:

$$solCOD = 6.73 + 0.074 * x_1 + 0.056 * x_2 \text{ (eq.5)}$$

Where $solCOD$ is expressed in % of total COD with standard deviation equal to 1.02, and the ranges for x_1 and x_2 are [7, 25] and [28, 96] respectively.

Soluble COD, which serves as an indirect measurement of the solubilization of particulate matter, could be expected to indicate the CH_4 potential of a substrate the hydrolysis of which is the limiting step. Experiment 3 that produced the highest CH_4 yield, showed also the maximum soluble COD though not that different from experiment 12 (*Table 1*). Additionally, experiments 7, 13 and 14 yielded similar CH_4 as experiment 12, besides having lower soluble COD values. According to *eq. 5*, the maximum soluble COD would correspond to harsh AAS conditions while the maximum CH_4 yield corresponded to milder conditions and resulted to be dependent on more factors (see section 3.1). Therefore, a lack of correlation among soluble COD and CH_4 yield can be hypothesized, a conclusion to which other studies in the field of anaerobic digestion have also reached (Tsapekos et al., 2015). Anaerobic digestion is a relatively slow process with HRTs of usually more than 15 days, and partly solubilizing the particulate matter might indicate improvement only of the initial conversion rate. On the other hand, the facilitated access to structural carbohydrates due to a pretreatment is not accounted for when considering only the initially solubilized fraction. Hence, only a slight effect on the structure of the biomass can result in an increased CH_4 yield without observation of significant solubilization. However, soluble COD is an interesting factor to take into account for the configuration of the process. For instance, in order to reduce the volume of the pretreated fibers that need to pass through an NH_3 removal step, a separation of the solids could be preferable. In such case, the soluble COD that would mainly remain in the liquid fraction may indicate the loss of biomass that will occur from such a separation, affecting the final CH_4 potential of the pretreated biomass. In this line, depending on the configuration of the pretreatment process, one could aim at maximizing the CH_4 yield while keeping the soluble COD at minimum levels. It is important to mention here that the models produced in this study express the specific system and might not be apt for describing a wider application of AAS to manure fibers of different origin. Nevertheless, these can be used for assessing general trends as well as the existence or not of interaction effects, information that can be valuable for the design of the process configuration.

3.3 Extension of Optimization experiments (fCCD)

Given that the optimal conditions for maximizing the CH_4 yield of AAS-treated fibers were found on the edge of the experimental area (minimum NH_3 concentration and maximum duration of AAS tested), a second optimization step took place where the ranges of the interacting parameters of AAS (NH_3 concentration and duration) were further investigated towards the optimum region following a faced CCD (fCCD). The S:L ratio was kept constant to the optimum value of 0.16 kg/l, as it was found not to interact with the rest of parameters.

Based on the cumulative CH_4 yield after 17 days of digestion of the second set of batch tests (*Table 2*), two new models were constructed for the experimental region covered by the fCCD (*eq.6* and *eq.7*). *Eq.6* corresponds to the empirical model constructed for predicting the cumulative CH_4 yield after 17 days of digestion of the AAS-treated fibers and *eq.7* to the prediction of the $CH_4\ yield\ increase$ as compared to the NP fibers. According to the ANOVA results for the constructed models, the most influencing effect in this range of AAS parameters was the NH_3 concentration of the reagent (x_1, *p =0.0006*), followed by the effect of the duration (x_2, *p =0.0110*) and lastly by the interaction effect of these parameters ($x_1 * x_2$, *p=0.0485*). The models were significant (*p = 0.0011*) and no lack-of-fit was detected (*p = 0.1770*). The fit of the experimental data to the models was found to be satisfactory ($R^2 = 0.85$) and in good agreement with the reduced models (*adjusted* $R^2 = 0.80$).

$$CH_4\ 17d = -52.409 + 33.809 * x_1 + 1.519 * x_2 - 0.203 * x_1 * x_2, \quad (eq.6)$$

$$CH_4 yield\ increase = -198.87 + 63.91 * x_1 + 2.87 * x_2 - 0.38 * x_1 * x_2, \quad (eq.7)$$

The high influence of the NH_3 concentration on the response can also be observed in *Fig.2b* where the Response Surface graph of *eq.6* is depicted; the CH_4 yield increases rapidly when the NH_3 concentration increases from 1 to 7% w/w. On the other hand, the duration of AAS appears to affect the CH_4 yield significantly mostly at low NH_3 concentrations, while at the maximum concentration tested in this design (7% w/w) the duration of AAS does not affect significantly the CH_4 yield when varied from 96 to 144 hours. This observation clearly indicates that the influence of the parameters depends greatly on the ranges chosen for optimization. Even though the duration that resulted at the maximum CH_4 yield would correspond to 144 hours, the experimental difference found in comparison to 96 hours was 3 ml CH_4/g TS (and 5 ml/g TS predicted by *eq.6*), making the difference insignificant. Thus, it appears that the optimal conditions of AAS as resulted from the 2nd set of optimization experiments correspond to the same optimum with the 1st set of experiments (cCCD), that is 7% w/w NH_3 and 96 hours. Based on *eq.6* a cumulative CH_4 yield of 193.43 ± 12.59 ml/g TS would result under these conditions, which is in line with the prediction of the first model (*eq.3*) at the same conditions. According to *eq.7*, this corresponds to a 265.92% ± 23.84 increase of CH_4 yield as compared to the yield of NP manure fibers. The prediction of *eq.7* lies closer to the values obtained experimentally as compared to the prediction of *eq.4*.

In order to validate the models, experiments under optimal conditions were repeated and the average CH_4 yield observed was 190.05 ± 6.70 ml/g TS, which is in line with the predictions of *eq.3* and *eq.6*. Taking into account all the experiments run under optimal conditions (cCCD, fCCD and Validation experiments) the average CH_4 yield observed was 198.95 ± 9.49 ml/g TS (274.56 ml/g VS), corresponding to a 243.73 % increase of the CH_4 yield after 17 days of digestion. Based on total COD measurements and assuming a CH_4 yield of 0.35 m^3/ kg O_2, it was calculated that the theoretical CH_4 yield of the manure fibers used in this study corresponded to 283.15 ml/g TS (421.66 ml/ g VS). Thus, considering a yield of 185.37 ml/g TS (255.81 ml/g VS) at STP conditions (0 °C, 1 atm) the AAS-treated manure fibers reached a 65.5 % of the theoretical CH_4 yield in only 17 days of digestion under optimal conditions.

Based on the results from both optimization designs, all three AAS parameters appeared to be influencing on the resulted CH_4 yield in some way. Generally the interaction among the duration of the pretreatment and the concentration of NH_3 was pronounced along the entire experimental region. It seems that in order to decrease the NH_3 concentration needed for a successful AAS process, the duration has to be increased and vice versa. In a large scale application though, it is more likely that the reagent concentration will limit the process configuration rather than the duration of AAS. High durations of pretreatment would be translated to an increased volume of pretreatment vessel, or to the need for additional pretreatment vessels running in parallel, affecting thus mainly the initial investment for the implementation of AAS. However, low reagent concentrations lead to easier handling, as well as to an easier target to fulfill in case a surplus of NH_3 is requested (Lymperatou et al., 2015b). On the other hand, the effect of the S:L ratio resulted to be independent of the other parameters, although highly significant. The highest S:L ratio tested in this study corresponded to the minimum volume of reagent in order to ensure the entire biomass was soaked. Thus a further increase would result to a partially pretreated batch, which is undesirable. On the other hand, the low S:L ratio is expected to be more expensive to perform, as the majority of the pretreatment mixture volume corresponds to the reagent. However, the NH_3 removal step of a mixture with low solids would probably be facilitated. All in all, the interaction of the

NH_3 concentration with the duration of AAS presents some flexibility on how the pretreatment can be applied.

3.4 Compositional changes of optimally-pretreated manure fibers

Ammonia, as an alkaline reagent, is known to produce partial delignification and occasionally swelling of the lignocellulosic structure. In the present study, no apparent delignification occurred on manure fibers, and the lignin content in both the control and the pretreated biomass accounted for ca. 16.3% TS (*Table 3*). Interestingly, when harsher conditions of AAS (32% w/w NH_3) were applied to swine manure fibers, a similar observation was reported (Jurado et al., 2013b). Delignification is often set to be the principal goal of a pretreatment as the bonding of lignin with hemicellulose and cellulose presents a barrier for enzymatic attack. Nonetheless, it has been reported that accessibility to cellulose is more important than actual removal of lignin for improving digestibility (Rollin et al., 2011). A correlation between lignin removal and temperature increase has been reported by different authors (Li et al., 2015; Yoo et al., 2013). The low temperature applied during AAS could partially explain why no lignin removal was observed in this study. However, AAS might affect differently each type of lignocellulosic biomass. For instance, (Antonopoulou et al., 2015) observed partial lignin removal in sunflower straw after application of AAS at ambient temperature, in contrast to grass and poplar where no apparent delignification occurred under the same conditions.

The cellulose fraction of the biomass also seems not to have been affected by AAS, as the glucan content of both biomasses (*Table 3*) was similar (assuming that all glucose was derived from cellulose). This is in line with the observations of other studies (Kim et al., 2008; Li et al., 2015). On the contrary, solubilization of hemicellulose was observed during the NH_3 treatment, as the xylan and arabinan contents were significantly reduced in the pretreated biomass (10.58 %TS and 3.10 %TS as compared to an initial 16.04 %TS and 5.71 %TS of xylan and arabinan respectively). This is also evident from the increase of soluble sugar concentration detected in the liquid fraction of the pretreated biomass, as well as from a significant increase of the acetic acid content (*Table 3*). The reduction of the insoluble organic N found in the pretreated biomass indicates that a slight solubilization of proteins might have occurred, whereas the significant increase of soluble organic N found (1.72% TS as compared to 0.80% TS in the NP fibers), could be attributed to the formation of nitrogenous compounds from the reaction of the NH_3-N reagent and the biomass. More studies have shown an increase of organic N in AAS-pretreated biomasses (Mirtsou-Xanthopoulou et al., 2014; Song et al., 2012). A description of possible reactions that can occur during NH_3-based pretreatments of lignocellulosic biomasses is presented in Chundawat et al., (2010). Further investigation of the fate of the reagent-N should be carried out in order to better understand how NH_3 interacts with the organic substances present in the manure fibers.

In conclusion, the mechanism of AAS appears not to be the same for all lignocellulosic biomasses. In comparison to the delignification effect of AAS on other biomasses, the AAS pretreatment appears to have a mild effect on manure fibers producing though surprisingly high increases of the CH_4 yield. A future systematic optimization of the same AAS parameters and ranges of different lignocellulosic biomasses for anaerobic digestion could further contribute on understanding the mechanism of this pretreatment on different lignocellulosic biomasses. Additionally, the identification of common characteristics of the biomasses that better respond to AAS could be assisted.

3.5 Comparison of pretreatments on increasing the CH_4 yield of manure fibers

Many different approaches have been tested so far for increasing the CH_4 yield of swine manure fibers. The majority of studies have focused on thermal pretreatments as these pose certain advantages such as short duration, inactivation of pathogens, and energy requirements can be reduced if the residual heat from associated Combined Heat and Power (CHP) plants is exploited. Raju et al. (2013) reported a 29% increase of CH_4 yield by thermal pretreatment of manure fibers at the range of 100 – 225 °C. Menardo et al. (2011) reported a 171% increase by pretreating swine manure fibers at 120 °C for 30 min. Ferreira et al. (2014) investigated the effect of thermal explosion of manure fibers under different combinations of temperature (120-180 °C) and duration (15-60 min), and demonstrated a 107% increase of CH_4 yield for 170 °C and 30 min pretreatment. Other pretreatments tested include mechanical, chemical, and biological processing; Hjørth et al. (2011) tested extrusion as a method for increasing the digestibility of the solid fraction of manure and reported an increase of 27% of cumulative CH_4 yield of pretreated manure fibers (both swine and cattle manure fibers). A biological pretreatment of fiber-rich swine manure for biogas production was reported to produce a 55% increase of CH_4 yield (Tuesorn et al., 2013). González-Fernández et al. (2008) compared the effectiveness of an acidic and an alkaline pretreatment and reported a negative effect of the acid to the CH_4 yield of pretreated fibers while the increase achieved by NaOH treatment was 13%.

From the work presented here, it appears that AAS has the potential to unlock the CH_4 potential of swine manure fibers at a great degree. Nevertheless, it is important to stress that not all pretreatments have been performed under optimal conditions. While a possible application of AAS of manure fibers in a larger scale would still need further investigation, especially in regards to the NH_3 recovery technology to be applied, a continuous process of manure mono-digestion enriched with AAS-treated fibers can further verify the efficiency of the process. While batch tests can facilitate optimization goals in regards to pretreatment conditions, experiments on continuous mode are more appropriate for evaluating real applications (Carrère et al., 2016). Previous work on a continuous anaerobic digester of manure enriched with manure fibers treated with 32% w/w NH_3 led to a 98% increase of CH_4 yield of manure fibers (Jurado et al., 2016). Based on the present study, AAS of manure fibers could further improve the CH_4 yield by using considerably milder conditions than previously thought.

Conclusions

Optimization experiments of AAS through RSM revealed a strong interaction among the NH_3 concentration and the duration of AAS. The optimal conditions of AAS at ambient temperature corresponded to 7% w/w NH_3 (aq.), 96 hours of AAS, and 0.16 kg fibers/l reagent, resulting to a 244% increase of the CH_4 yield in only 17 days of batch digestion. The degree of solubilization of the biomass increased with increased severity of AAS and compositional analyses showed that significant solubilization of hemicellulose occurred during optimized AAS, while no delignification or loss of the cellulose fraction was observed.

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Table 1

Experimental conditions and results of soluble COD, cumulative CH_4 yields and CH_4 yield increase of AAS-treated fibers from the cCCD experiments

N° of experiment	NH_3 concentration (% w/w)	Duration of AAS (hours)	S:L ratio (kg fibers/l reagent)	% soluble COD	CH_4 yield 17d (ml CH_4/g TS)[1]	% increase CH_4 yield 17d[1]
1	7	28	0.16	8.31	126.64 ± 11.52	101.31 ± 18.31
2	25	28	0.16	10.75	159.56 ± 8.11	153.65 ± 12.89
3	7	96	0.16	14.62	213.96 ± 15.04	240.12 ± 23.91
4	25	96	0.16	12.53	173.23 ± 5.40	178.78 ± 8.59
5	7	28	0.28	9.47	126.84 ± 3.55	101.63 ± 5.65
6	25	28	0.28	9.54	160.87 ± 4.89	155.73 ± 7.78
7	7	96	0.28	11.89	176.27 ± 3.93	180.21 ± 6.24
8	25	96	0.28	13.39	162.47 ± 3.90	158.27 ± 6.20
9	0.9	62	0.22	8.89	130.06 ± 2.28	106.74 ± 3.62
10	31.1	62	0.22	13.17	173.60 ± 5.47	175.97 ± 8.70
11	16	4,8	0.22	7.25	117.38 ± 4.49	86.59 ± 7.13
12	16	119.2	0.22	14.17	174.29 ± 8.74	177.07 ± 13.90
13	16	62	0.12	12.17	179.05 ± 6.28	184.63 ± 9.98
14	16	62	0.32	12.99	178.50 ± 9.98	183.72 ± 15.86
15	16	62	0.22	12.16	170.74 ± 5.17	171.41 ± 8.22
16	16	62	0.22	11.80	159.66 ± 6.23	153.80 ± 9.90
17	16	62	0.22	10.63	153.11 ± 8.27	143.39 ± 13.15
18	16	62	0.22	10.85	143.15 ± 6.40	127.56 ± 10.18
19	16	62	0.22	11.69	155.48 ± 12.84	147.16 ± 20.41
NP	-	-	-	0.12	62.91 ± 3.49	-

[1] Values correspond to average values from triplicates ± the standard deviation.

Table 2

Cumulative CH_4 yields and CH_4 yield increase of AAS-treated fibers from the fCCD as resulted after 17 days of digestion

N° of experiment	NH_3 concentration (% w/w)	Duration of AAS (hours)	CH_4 yield 17d (ml CH_4/g TS)[1]	% increase CH_4 yield 17d[1]
1	1	96	104.17 ± 7.07	97.06 ± 13.38
2	7	96	192.85 ± 6.73	264.84 ± 12.72
3	1	144	163.23 ± 6.04	208.80 ± 11.43
4	7	144	193.41 ± 10.47	265.90 ± 19.80
5	1	120	135.75 ± 6.94	156.82 ± 13.14
6	7	120	186.19 ± 8.51	252.24 ± 16.11
7	4	96	136.14 ± 8.35	157.55 ± 15.80
8	4	144	178.07 ± 5.66	236.88 ± 10.72
9	4	120	180.85 ± 8.42	242.13 ± 15.93
10	4	120	190.09 ± 4.98	259.61 ± 9.42
11	4	120	178.86 ± 11.99	238.36 ± 22.67
12	4	120	170.45 ± 8.89	222.46 ± 16.82
NP	-	-	52.86 ± 11.47	-

[1] Values correspond to average values from triplicates ± the standard deviation.

Table 3

Composition of raw manure fibers (NP) and optimally AAS-treated manure fibers[1]

Composition	NP manure fibers (% TS)	AAS-treated manure fibers (% $TS_{initial}$)
Glucan	27.20 ± 0.14	27.88 ± 1.03
Xylan	16.04 ± 0.28	10.58 ± 0.45
Arabinan	5.71 ± 0.2	3.10 ± 0.22
Total structural carbohydrates	48.95 ± 0.34	41.57 ± 1.69
Acid-insoluble lignin	16.30 ± 0.68	16.35 ± 0.81
Acid-soluble lignin	0.33± 0.00	0.38 ± 0.00
Extractives & volatiles	9.10 ± 0.17	12.88 ± 2.70
Free sugars	0.09 ± 0.00	0.09 ± 0.03
Soluble sugars	0.08 ± 0.00	0.68 ± 0.03
Acetic Acid	0.03 ± 0.00	0.68 ± 0.01
NH_4^+-N content	0.37 ± 0.00	0.98 ± 0.00
Soluble Organic N	0.80 ± 0.14	1.72 ± 0.06
Non-soluble organic N	1.61 ± 0.01	1.45 ± 0.06
C/N ratio	14.37 ± 0.25	11.85 ± 1.01
Total Ash	32.62 ± 1.34	29.81 ± 1.52
Solid Recovery	-	98.65

[1] Values correspond to average values from replicates ± the standard deviation. Solid Recovery was calculated as g TS after pretreatment divided by the g TS before treatment and multiplied with 100. C/N ratio and Solid recovery values are unitless.

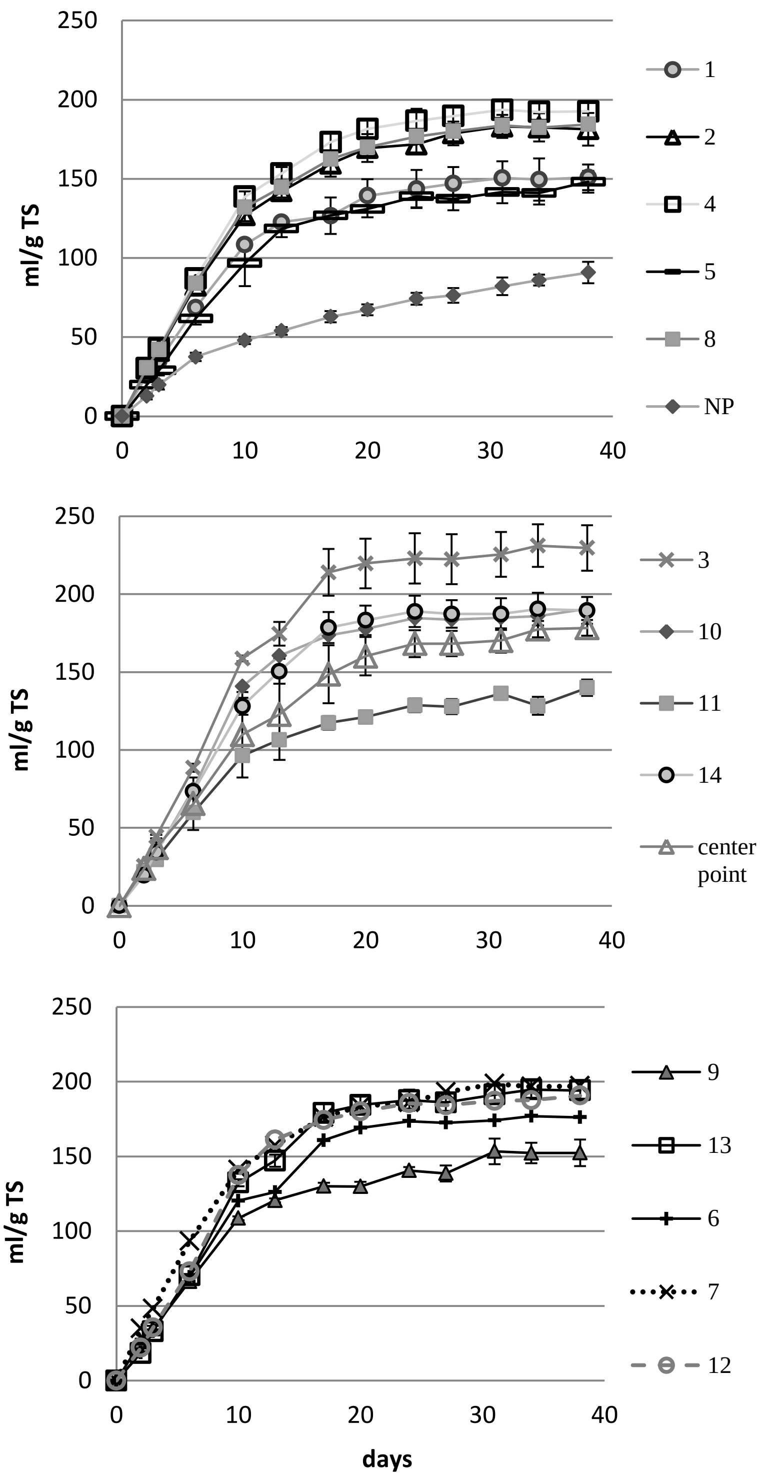

Fig. 1 Cumulative CH_4 yields of BMP tests of manure fibers treated under different conditions of AAS according to the circumscribed CCD (*Table 1*). Center point corresponds to the average of experiments 15-19. Vertical bars correspond to standard deviation of triplicates.

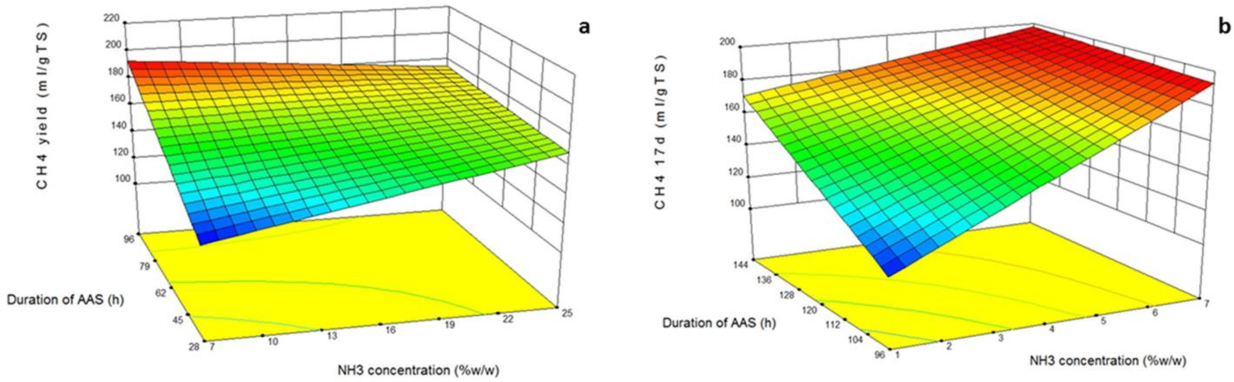

Fig. 2 Response surface graphs of a) 1st (cCCD) and b) 2nd (fCCD) set of optimization experiments. The cumulative CH_4 yield of swine manure fibers is plotted as a function of the ammonia concentration and the duration of AAS.

Paper IV:

"Optimization of Aqueous Ammonia Soaking at ambient temperature for Enhancing the Methane Yield of Wheat Straw"

"Optimization of Aqueous Ammonia Soaking at ambient temperature for Enhancing the Methane Yield of Wheat Straw"

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Abstract

Aqueous Ammonia Soaking (AAS) at ambient temperature was applied to wheat straw under different conditions in order to maximize the CH_4 yield through mesophilic anaerobic digestion. The effects of the NH_3 concentration, duration of AAS and solid-to-liquid ratio were studied on the resulting CH_4 yield and the solubilization degree of the pretreated wheat straw. A strong interaction among NH_3 concentration and duration of AAS was observed. The optimal conditions found were 18 % w/w NH_3, 7 days of duration and 50 g straw/ l reagent, leading to a 43 % increase of the CH_4 yield in 17 days of digestion. Compositional analysis of the optimally-treated wheat straw revealed that a significant solubilization of hemicellulose took place during AAS together with a moderate lignin removal. This study points to the necessity for further investigation on the fate of the reagent-derived N, as well as a techno-economic analysis coupling AAS at low temperature with an NH_3 recovery process for assessing the feasibility of the pretreatment on wheat straw.

Keywords: wheat straw, pretreatment, anaerobic digestion, optimization, aqueous ammonia soaking

1. Introduction

A greener energy production system is forthcoming, as numerous countries worldwide have set goals for achieving a shift of part of the energy production from fossil fuels to renewable energy sources in the near future. Specifically, there is a target of achieving a 20% share of the energy consumption to originate from renewable resources until 2020 in the EU [1]. Bioenergy is expected to cover 60% of the total renewable energy production until then [2]. In this line, agricultural residues such as straw constitute an important source for energy production, due to their high availability. Among other applications, straw has been traditionally used in animal breeding as feed and as bedding material, as well as incorporated to the soil for reducing erosion risk and improving soil fertility [3]. Taking into account the amount of residues used for sustainable agricultural practices, as well as competitive uses of these residues, Scarlat et al. [3] have calculated the energy potential of crop residues in Europe to reach 1537 PJ on average annually. In Europe the most abundant agricultural straw originates from wheat crop, which is also the second most abundant worldwide [4]. The conversion of wheat straw into energy is possible through various processes, such as combustion, gasification, fermentation to produce ethanol and anaerobic digestion (AD) for biogas production [5].

Among the renewable energy products, biogas is expected to contribute with a 12% share of the European bioenergy platform of 2020 [2]. Wheat straw has been identified as a suitable co-substrate to other organic residues that are usually sent to AD plants e.g. livestock manure [6–9], as it can improve the C/N ratio, boost the biogas production and increase the dry matter loading of liquid waste streams. An additional advantage of using wheat straw as a substrate for AD is that the carbon remaining in the digestate can be returned to the soil, reducing this way direct competition with the practice of incorporating straw to the soil. The digestion of wheat straw for biogas production has been studied in the past under both mesophilic and thermophilic conditions and with various sources of inoculum [10–13]. Nevertheless, the conversion to biogas is limited due to the rigid lignocellulosic structure of wheat straw that restricts access to microbial enzymes. This fact has resulted in numerous studies employing pretreatment strategies in order to increase the digestibility of wheat straw.

Aqueous ammonia soaking (AAS) is identified as a pretreatment with a great potential to be applied on lignocellulosic biomasses for improving their conversion to biofuels. Ammonia is known to cause delignification or swelling of lignocellulose, rendering this way the biomass more easily convertible to the desired products. However, the main advantage of AAS is the possibility of recovering and reusing ammonia, avoiding thus the addition of chemicals for pH correction of the pretreated biomass. As a result, the cost of chemicals of the pretreatment is reduced. Extensive work has been carried out lately on AAS of different lignocellulosic biomasses for improving ethanol [14] and biogas production [11,15–23]. Particularly in the case of wheat straw, ammonia pretreatments have been tested in the past for improving the production of biogas [11,20,22]. Nevertheless, in these studies the ammonia pretreatment was performed under relatively high temperatures. AAS at ambient temperature can present certain advantages over other configurations of ammonia treatment [24]. Usually temperature less than 80°C is considered not to involve extra energy input, as the waste heat from the gas engines can be used [22]. Nevertheless, in a chemical pretreatment where NH_3 is expected to be recovered, the waste heat could be more efficiently used when applied to the end of the pretreatment for facilitating the NH_3 recovery process. Moreover, when thermal alkaline pretreatments are applied on lignocellulosic biomasses, there is an increased risk of formation of inhibitory by-products [25]. Although AD is less sensitive to these inhibitors than fermentation, an estimation of such effect was not possible in previous studies as the pretreated straw was washed and only the solid fraction was digested [22]. This approach can also lead to a reduced methane yield due to the organic fraction that is solubilized. All in all, the expected low energy consumption (given ambient temperature and pressure is applied), in combination with the possibility of recovering ammonia, makes AAS a good candidate for application to wheat straw, minimizing the cost of implementation at a large scale. Previous experiments of AAS at ambient temperature (where both liquid and solid fractions were used for digestion) have shown that the methane yield of the pretreated biomass was significantly higher than the untreated [18]. However, the pretreatment was tested only under one set of conditions and a further investigation of the effects of the AAS parameters could give an insight on the different possible configurations of the pretreatment.

In this study, the effect of the AAS parameters on the resulting short-term and ultimate methane yield of pretreated wheat straw were investigated, as well as the effect on the solubilization and the hydrolysis rate. The optimal pretreatment conditions at ambient temperature leading to the highest conversion of wheat straw to methane in short term were assessed. Response Surface Methodology was applied and empirical models were produced for predicting the methane yield and the degree of solubilization of the treated straw under the conditions tested.

2. Materials and Methods

2.1 Substrate and Inoculum

The wheat straw used for the experiments was harvested from Sjæland region in Denmark and was stored in big bales in a dry and dark room. Prior to use, it was milled to 6mm by a cutting mill (Retsch SM 2000, Germany). The total solids (TS) and Volatile Solids (VS) content of the wheat straw were 93.75 ± 0.22% and 89.21 ± 0.55% of wet mass respectively. The total Chemical Oxygen Demand (COD) of the wheat straw was 1.25 ± 0.14 g O_2/ g VS, based on which the theoretical CH_4 yield was calculated to be 436.33 ml/ g VS, by assuming 350 ml CH_4 /g COD. The inoculum used for the Biochemical Methane Potential (BMP) tests originated from a centralized full-scale biogas plant digesting livestock manure and organic waste under mesophilic conditions (Hashøj Biogas, Denmark). The inoculum was incubated at 37°C for 10 days prior to use for reducing the residual biogas production. The main characteristics of the inoculum were 2.77% TS, 1.49% VS, 2.86 g NH_4^+-N /l, 8.04 g soluble COD/l, and pH 8.0.

2.2 Aqueous Ammonia Soaking (AAS) pretreatment

The different pretreatment batches of wheat straw took place in 2 l screw-capped laboratory bottles. The solution of aqueous ammonia (reagent) was added and the bottles were sealed for avoiding losses of ammonia reagent. The mixture was left intact (no mixing) until the end of the pretreatment. At the end of the pretreatment duration, the NH_3 was removed by vacuum evaporation (Buchi Rotavapor, Switzerland) at 130 mbar while the temperature of the water bath was progressively increased up to 80°C. Prior to the evaporation step, an equal volume of tap water was added to the mixture for facilitating the handling of the biomass and the vacuum evaporation of NH_3. The final concentration of NH_4^+-N in all batches after evaporation was less than 0.8 g /l securing thus that no inhibitory effect would occur during the subsequent AD step.

2.3 Biochemical Methane Potential (BMP) tests

The BMP tests were set with 1.0 g TS of wheat straw and 40 ml of inoculum in 320 ml infusion bottles. Due to difficulties with representative sampling of the pretreated straw, the pretreated biomass was separated to a solid and a liquid fraction. Subsequently, the respective amount of the solid and liquid fraction was added to the BMP bottle based on the mass/volume (m/v) ratio of the initial pretreated batch. The BMP tests were set up in two blocks. In each block of experiments, two additional BMP tests were set up, one with inoculum and raw wheat straw, used as control, and one only with inoculum used as blank. All BMP tests were set at triplicates. The bottles were flushed with a mixture of 80% N_2/20% CO_2, sealed with rubber stoppers, secured with aluminum crimps and placed in an incubator at 37°C. The CH_4 production was monitored periodically until the end of the experiments. The CH_4 production of the BMP tests of both pretreated and raw wheat straw was corrected for the residual production of the inoculum by subtracting the CH_4 production of the blank tests. Values of CH_4 yield reported are given at 20°C, unless otherwise mentioned and correspond to average yields of triplicates along with the standard deviation. The hydrolysis rate is considered to be the limiting step in anaerobic digestion of lignocellulosic substrates and first order kinetics can thus be used for describing the hydrolysis process [26]. The hydrolysis rates k of the BMP tests were calculated as described in [26] by applying equation 1 to each BMP test.

$$B = B_0 * (1 - e^{-kt}), \text{(eq.1)}$$

where B and B_0 correspond to the CH_4 yield after t days of digestion and the ultimate CH_4 yield of the sample added, respectively. The criterion for choosing the duration t for each BMP was that the methane production had reached the 65% of the ultimate value, $B = 0.65 * B_0$.

Table 1 Levels of parameters based on the Central Composite Design followed for the optimization of AAS of wheat straw

AAS Parameter	Low level	Center level	High level
	-1	**0**	**1**
A -NH_3 concentration (% w/w)	1	16.5	32
D - Duration of AAS (days)	1	4	7
SL - Solid-to-liquid ratio (g straw/l reagent)	50	75	100

2.4 Experimental Design and data analysis

In order to study the effects of the AAS parameters on the CH_4 yield of pretreated wheat straw, a faced Central Composite Design (CCD) was followed and the results were analyzed by Response Surface Methodology. The software used for the analysis was Design Expert 9.0.6.2 (Stat-Ease, USA). Three parameters of AAS were chosen as independent variables, namely the NH_3 concentration of the reagent, the duration of AAS and the solid-to-liquid (S:L) ratio. This approach permitted following a structured experimental design where the 3 parameters were set at 3 different levels (Table 1) and the main and quadratic effects of each parameter as well as any interaction effects among the latter could be estimated with a relatively small number of experiments, by taking advantage of the geometry of the experimental region. Additionally, one set of conditions (center point) was repeated 6 times in order to estimate the variation of the response including all experimental errors. Finally, an empirical equation was constructed, where the response is expressed as a function of the significant effects as detected from the statistical analysis. This equation has the following general form:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad \text{(eq.2)}$$

Where

Y, is the dependent parameter (response);

x_i , i=1,2,3 are the independent parameters;

b_0 is the intercept coefficient;

b_i , i=1,2,3 are the regression coefficients expressing the main effect of each parameter on the response;

bij, i,j=1,2,3, j≠i, are the regression coefficients for the interaction effect of 2 independent parameters on the response;

b_{ii}, i=1,2,3, are the regression coefficients for the quadratic effect of each independent parameter on the response.

The regression coefficients were calculated by regression analysis of the experimental data. The model obtained was assessed by ANOVA and statistical significance was tested by Fisher's F-test. All terms expressing main effects were included in the final models, while only interaction and quadratic effects found statistically significant (p value < 0.05) were included. Based on the empirical model obtained, a Response Surface graph was constructed where the predicted response was plotted as a function of two independent parameters in a three-dimension graph.

2.5 Compositional analysis

Compositional analysis was performed on both raw wheat straw and optimally AAS-treated wheat straw. All analyses were performed at least in duplicates and the average values are reported accompanied with the standard deviation. The pretreated wheat straw was dried at 45°C and both raw and dried pretreated biomasses were grounded to 1mm by a laboratory grinder (IKA, MF 10.1, IKA®-Werke GmbH). The biomasses were subjected to a two-step extraction process, first with Millipore-grade water for 6 hours and subsequently with 96% v/v ethanol for 24 hours, according to [27]. The extracted biomasses were used for determination of structural carbohydrates and lignin (acid-insoluble and acid-soluble) following the two step acid hydrolysis procedure according to [28]. Acid-soluble lignin was measured at 205 nm and the absorptivity ε (35.94 L/g·cm) was determined following NREL's protocol [29].

Determination of soluble components of raw wheat straw was carried out by soaking the straw in water for 24 hours while for the pretreated straw, the liquid fraction after the ammonia removal step was used. Both mixtures (water-straw and pretreated straw) were centrifuged at 10,000 rpm for 10 min and subsequently filtered to pass through a 0.45 μm membrane filter. Free sugars were determined in the liquid samples and oligosaccharides were determined after a dilute-acid hydrolysis step as described in [30]. Free sugars were determined after acidifying liquid samples with 0.1 M H_2SO_4 and filtering through 0.22 μm membrane filters. Both raw and pretreated biomasses were analyzed for their composition in C, H, N, O, by an Elemental analyzer (EuroVector, Model EA 3000). Acetanilide was used as a standard.

2.6 Analytical Methods

TS, VS and ash determination was carried out according to [31]. NH_4^+-N and soluble COD determination was performed after the NH_3 evaporation step by Hach Lange kit LCK 305 and LCK 514 respectively; samples of the pretreated straw were centrifuged at 10,000 rpm for 10 min and filtered through 0.45 µm. For the total COD determination, the wheat straw was milled to powder with a commercial coffee grinder and diluted with Millipore-grade water. All determinations were based on triplicates. Measurement was done by Hach Lange kit LCK 514. Carbohydrate and acetic acid quantification was performed by High Performance Liquid Chromatography (HPLC) with a refractive index and an Aminex HPX-87H column (BioRad) at 63°C. A solution of 12mM H_2SO_4 was used as eluent at a flow rate of 0.6 ml/min. The detection and quantification of CH_4 was performed by Gas Chromatography (GC) with a Porapak Q packed column (6 ft. and I.D. 3 mm) and a Thermal Conductivity Detector (TCD) and N_2 was used as carrier gas. The temperature of the injector, the oven and the detector were all set at 70 °C.

3. Results and Discussion

3.1 Effect of AAS parameters on solubilization of COD

Twenty batches of wheat straw were pretreated with AAS based on the Central Composite Design (CCD) followed, as shown in Table 3. The degree of COD solubilization due to the pretreatment varied from 7.27 to 23.60% of total COD of the wheat straw. Modelling (by Response Surface Methodology, RSM) the fraction of COD solubilized as a response of the AAS parameters, showed (Table 2) that mainly the NH_3 concentration ($p < 0.0001$) was responsible for the extent of solubilization, followed by the duration of AAS ($p = 0.0025$), while the S:L ratio was found to be statistically insignificant ($p > 0.0500$). The model was highly significant ($p < 0.0001$) and no lack of fit was detected ($p = 0.8548$). The R^2 (0.82) was in good agreement with the adjusted R^2 (0.78), indicating that the terms used in the model were sufficient for describing the response. Both influencing factors had a positive correlation with the fraction of COD solubilized. The final expression of the % of COD solubilized as a function of the AAS parameters was according to equation 3.

$$\%\ sol\ COD = 4.20 + 0.91 * A + 0.94 * D + 0.02 * A^2\ (eq.3)$$

Where:

$\%\ sol\ COD$, the fraction of total COD solubilized after AAS

A, the NH_3 concentration of the reagent in % w/w

D, the duration of AAS in days

Table 2 ANOVA table of empirical model predicting % COD solubilized by AAS (eq.3)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Block	12.17	1	12.17		
Model	407.97	3	135.99	22.51	< 0.0001
A-Ammonia	252.52	1	252.52	41.80	< 0.0001
B-Duration	79.76	1	79.76	13.20	0.0025
A^2	75.69	1	75.69	12.53	0.0030
Residual	90.63	15	6.04		
Lack of Fit	51.08	11	4.64	0.47	0.8548
Pure Error	39.54	4	9.89		
Cor Total	510.77	19			

Terms found to be statistically non-significant and excluded from the model: SL (p = 0.2430), A·D (p = 0.3684), A·SL (p = 0.7198), D·SL (p = 0.5827), D^2 (p = 0.1787), SL^2 (p = 0.6868).

From previous studies on lignocellulosic biomasses, it is known that AAS often results in solubilization of lignin and hemicellulose [14]. Thus, including the soluble fraction of the pretreated biomass in anaerobic digestion tests may result to an increase or decrease of the product yield, as this may contain valuable organic matter or inhibitory by-products. Severe inhibition though is not that likely in AD as the usual degradation products of pretreated lignocellulosic biomasses such as HMF, furfurals, lignin polymers and derivatives have been shown not to cause severe inhibition [32]. Consequently and, depending on the conditions of AAS a significant loss of biogas or CH_4 could occur if only the solid fraction is used for digestion.

3.2. Effect of AAS parameters on the hydrolysis rate of wheat straw

The BMP experiments lasted 59 days after which no significant CH_4 production was observed for the pretreated wheat straw. The CH_4 production curves of the BMP tests are shown in Fig.1. The AAS pretreatment affected positively the hydrolysis rate in all cases (considering the hydrolysis rate to be the limiting step in anaerobic digestion of straw). The BMP tests of the pretreated wheat straw reached the 65% of their ultimate CH_4 yield within 10-14 days of digestion and the raw wheat straw within 17-21 days of digestion. The raw wheat straw averaged a rate of 0.0655 d^{-1}, while the rate of the pretreated straw ranged from 0.0802 d^{-1} to 0.1346 d^{-1}. The high R^2 of the fit of the experimental data confirms that assuming 1st order kinetics is justified (Table 3). The highest increase of hydrolysis rate was more than twice as large as that of the raw biomass and corresponded to experiment 14, where AAS was applied at 32% w/w of NH_3 concentration and at the center levels of duration and S:L ratio. Nevertheless different biodegradability degrees have been also obtained among batches, resulting to different ultimate CH_4 yields. Consequently, the highest hydrolysis rate does not correspond to the experiment with the highest short term CH_4 yield.

Thus, the hydrolysis rates should be interpreted in combination to the ultimate CH_4 yields obtained under certain conditions.

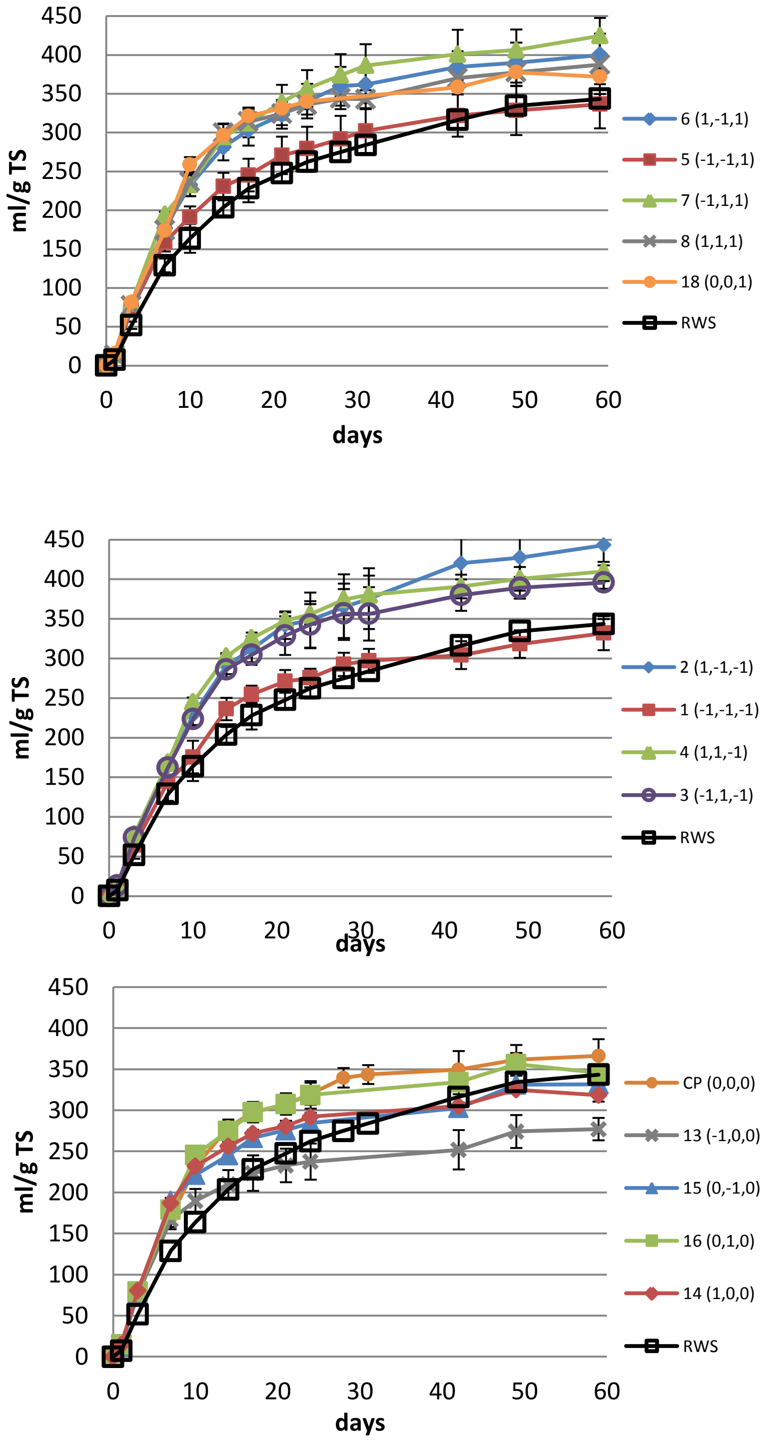

Figure 1 Graph of cumulative methane yields of BMP tests of AAS-treated wheat straw under different pretreatment conditions during digestion experiments. Numbers of experiments correspond to different AAS conditions explained in Table 2 and numbers in

parenthesis correspond to the coded values of the design as shown in Table 1. Points correspond to average values from triplicates and vertical bars to the standard deviation.

3.3 Effect of AAS parameters on CH_4 yield of wheat straw

Table 3 summarizes the results of the ultimate CH_4 yield of all batches obtained from the batch experiments. The raw wheat straw used in this study produced an average of 343.44 ml/g TS. The pretreatment affected the ultimate CH_4 yield in different ways when applied under different conditions. The range of cumulative CH_4 yield of the AAS-pretreated wheat straw varied from 276.96 ml/g TS to 443.01 ml/g TS, producing thus a positive and a negative effect on the digestibility of the biomass depending on the conditions. An empirical model was constructed in order to predict the ultimate CH_4 yield of AAS-treated wheat straw as a function of the influencing conditions of the pretreatment (eq.4).

$$ultCH_4\ yield = 553.21 + 9.70 * A + 12.76 * D - 8.46 * SL - 0.53 * A * D - 0.03 * A * SL - 0.12 * A^2 + 0.06 * SL^2 \quad \text{(eq.4)}$$

Where:

$ultCH_4\ yield$, the cumulative CH_4 yield of AAS-treated straw at the end of digestion expressed in ml/ g TS

A, the NH_3 concentration of the reagent, expressed in % w/w in water

D, the duration of AAS expressed in days

SL, the solid-to-liquid ratio of the pretreatment mixture, expressed in g wheat straw/ l reagent

Table 3 Experimental conditions and results of wheat straw pretreated with AAS under different conditions following the CCD

N° of experiment	NH_3 concentration (% w/w)	Duration of AAS (days)	Solid-to Liquid ratio (g/l)	COD solubilized (% total COD)	CH_4 yield 17d (ml CH_4/g TS)	% increase CH_4 yield 17d	Ultimate CH_4 yield (ml CH_4/g TS)	Hydrolysis rate (d^{-1})
1	1	1	50	7.18	254.67 ± 10.95	18.1	332.06 ± 21.36	0.0802 (0.992)
2	32	1	50	18.27	320.60 ±1 5.99	44.6	443.01 ± 25.42	0.0814 (0.995)
3	1	7	50	11.75	304.45 ± 12.51	41.2	395.78 ± 7.62	0.0915(0.992)
4	32	7	50	21.70	325.59 ± 6.96	51.0	409.93 ± 12.08	0.0967 (0.990)
5	1	1	100	7.27	245.30 ± 21.02	13.8	336.55 ± 31.24	0.0849 (0.996)
6	32	1	100	12.49	303.18 ± 19.85	40.6	400.02 ± 27.62	0.0896 (0.998)
7	1	7	100	9.34	312.26 ± 18.28	44.8	424.74 ± 22.84	0.0858 (0.996)
8	32	7	100	22.50	313.92 ± 13.53	45.6	387.53 ± 14.59	0.1057 (0.989)
9	16.5	4	75	23.60	291.00 ± 20.32	35.0	364.52 ± 15.00	0.0879 (0.992)
10	16.5	4	75	17.04	296,56 ± 28.10	37.6	372.19 ± 33.88	0.0972 (0.998)
11	16.5	4	75	15.80	295.14 ± 8.57	36.9	375.04 ± 3.96	0.0975 (0.996)
12	16.5	4	75	17.84	315.29 ± 12.29	46.2	395.81 ± 11.93	0.0986 (0.987)
13	1	4	75	9.04	223.40 ± 21.53	-7.3	276.96 ± 18.90	0.1231 (0.988)
14	32	4	75	19.87	271.96 ± 4.46	12.9	318.49 ± 13.68	0.1346 (0.994)
15	16.5	1	75	11.54	266.42 ± 4.71	10.6	331.75 ± 10.30	0.1175 (0.989)
16	16.5	7	75	19.69	297.71 ± 12.34	23.6	345.52 ± 8.38	0.1241 (0.986)
17	16.5	4	50	19.90	293.42 ± 13.75	21.8	355.25 ± 6.00	0.1060 (0.985)
18	16.5	4	100	17.15	321.29 ± 11.19	33.4	371.91 ± 9.42	0.1162 (0.971)
19	16.5	4	75	20.73	301.25 ± 4.55	25.1	353.94 ± 18.90	0.1199 (0.986)
20	16.5	4	75	18.01	285.51 ± 1.77	18.5	336.61 ± 7.73	0.1270 (0.992)
RWS 1	-	-	-	-	215.60 ± 5.77	-	347.78 ± 26.97	0.0573 (0.996)
RWS 2	-	-	-	-	240.91 ± 7.21	-	339.10 ± 13.31	0.0737 (0.997)

RWS stands for raw wheat straw. Experiments from 1 to 12 and RWS 1 correspond to the 1st block of BMPs, and experiments from 13 to 20 and RWS 2 correspond to the 2nd block. Values for CH_4 yields reported correspond to average yields with the standard deviation of triplicates. Numbers in parentheses next to hydrolysis rates correspond to the R^2 of the fit of experimental data to the 1st order equation.

Table 4 ANOVA table of experimental results from the BMP tests after 59 days of digestion

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Block	12056.67	1	12056.67		
Model	15852.75	7	2264.68	13.29	0.0001
A-Ammonia	3720.66	1	3720.66	21.83	0.0007
D-Duration	1442.64	1	1442.64	8.46	0.0142
SL-S:L ratio	23.10	1	23.10	0.14	0.7197
A*D	4874.79	1	4874.79	28.60	0.0002
A*SL	1221.17	1	1221.17	7.17	0.0215
A^2	2504.48	1	2504.48	14.70	0.0028
SL^2	4205.15	1	4205.15	24.67	0.0004
Residual	1874.71	11	170.43		
Lack of Fit	1188.05	7	169.72	0.99	0.5377
Pure Error	686.66	4	171.67		
Cor Total	29784.13	19			

Terms found to be statistically non-significant and excluded from the model: D·SL ($p = 0.2333$), D^2 ($p = 0.2625$)

As shown in Table 4, the model constructed was found to be highly significant ($p = 0.0001$) and no lack of fit was detected ($p = 0.5377$). The R^2 (0.89) was in good agreement with the adjusted R^2 (0.83) of the reduced model (insignificant parameters were left out). Among the three AAS parameters tested, the NH_3 concentration had the most significant influence on the response as the main (A) and quadratic (A^2) effects were found to be highly significant as well as both interaction terms with NH_3 (A·D, A·SL). Apart from the interaction with NH_3 concentration, the duration of AAS had a significant main effect (D) and the S:L ratio a significant quadratic effect (SL^2).

The interaction between the NH_3 concentration and the duration of AAS was found to be the most influencing on the ultimate CH_4 yield. The response surface graphs presented in Fig.2 show how the prediction of the ultimate CH_4 yield is affected by varying these two parameters at the three different levels of S:L ratio. Based on the surface graphs, it is clear that a reduction of the NH_3 concentration used for the pretreatment has to be compensated by a longer duration. Interestingly, when both parameters are set at the lowest or at the highest levels the efficiency of the pretreatment is reduced. Thus, harsh conditions are not in favor of an enhanced bioconversion of wheat straw. This is in agreement to other studies of AAS on lignocellulosic biomasses for improving bioconversion [33]. The dark blue regions of the surfaces (the reader is referred to the colored web version) correspond to ultimate CH_4 yields equal to or lower than the yield of the raw wheat straw. In a previous study [22], when NH_3 was applied to wheat straw with heat application (32.2 – 67.8°C), the interaction among NH_3 concentration and duration was found not to be significant on the resulted biogas yield, and in fact the duration of AAS was reported to be the least important factor among temperature, NH_3 concentration and duration of AAS. This can be explained due to the low range of duration tested in that study (up to 48 hours), as well as due to the strong interaction among temperature and NH_3 concentration that could have covered the effect of the duration.

A comparison among the surface graphs at different S:L ratios (Fig.2) also shows the quadratic effect of this factor, which expresses that the ultimate CH_4 yield is increased when the lowest or the highest S:L ratio is applied, while the middle S:L ratio (75 g/l) results to the poorest CH_4 yield regardless the rest of conditions. This is also evident from Fig.3 where the ultimate CH_4 yield is plotted as a function of the S:L ratio and the NH_3 concentration. Not many studies of AAS have included the effect of the S:L ratio. Nevertheless, when included as an influencing parameter of AAS on biomasses it has been reported that a decrease of the S:L ratio is linked to an increased enzymatic digestibility or ethanol yield [34–36]. The main effect of the duration of AAS can be observed in Fig.3, where the response surface is elevated as duration increases from 1 to 4 and to 7 days. Due to the interaction of the duration and the NH_3 concentration, this is more pronounced at low NH_3 concentrations. When duration is minimum (1 day), then the conditions of AAS that result to an enhanced CH_4 yield are narrowed to the maximum NH_3 concentration (32 % w/w) and the minimum S:L ratio (50 g/l). These conditions are found to be the optimal for maximizing the final digestibility of wheat straw. The importance of the duration of AAS for increased biomass digestibility has been observed in more studies [34,37].

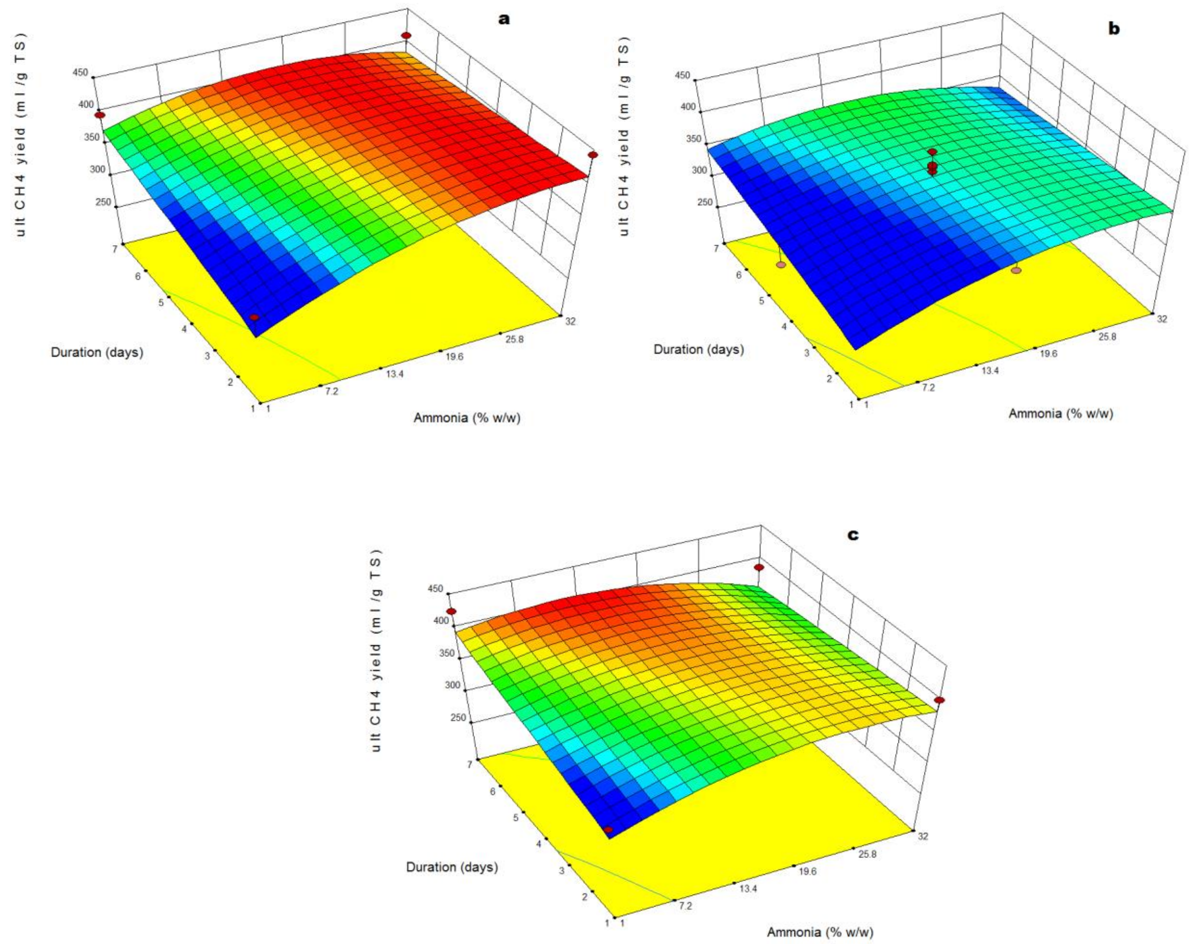

Figure 2. Response Surface graphs based on equation 4. The predicted ultimate CH_4 yield of AAS-treated wheat straw is plotted as a function of the NH_3 concentration and the duration of AAS. The S:L ratio is set constant in each graph, corresponding to 50, 75 and 100 g/l in a, b and c respectively. Dots in figures correspond to the experimental points.

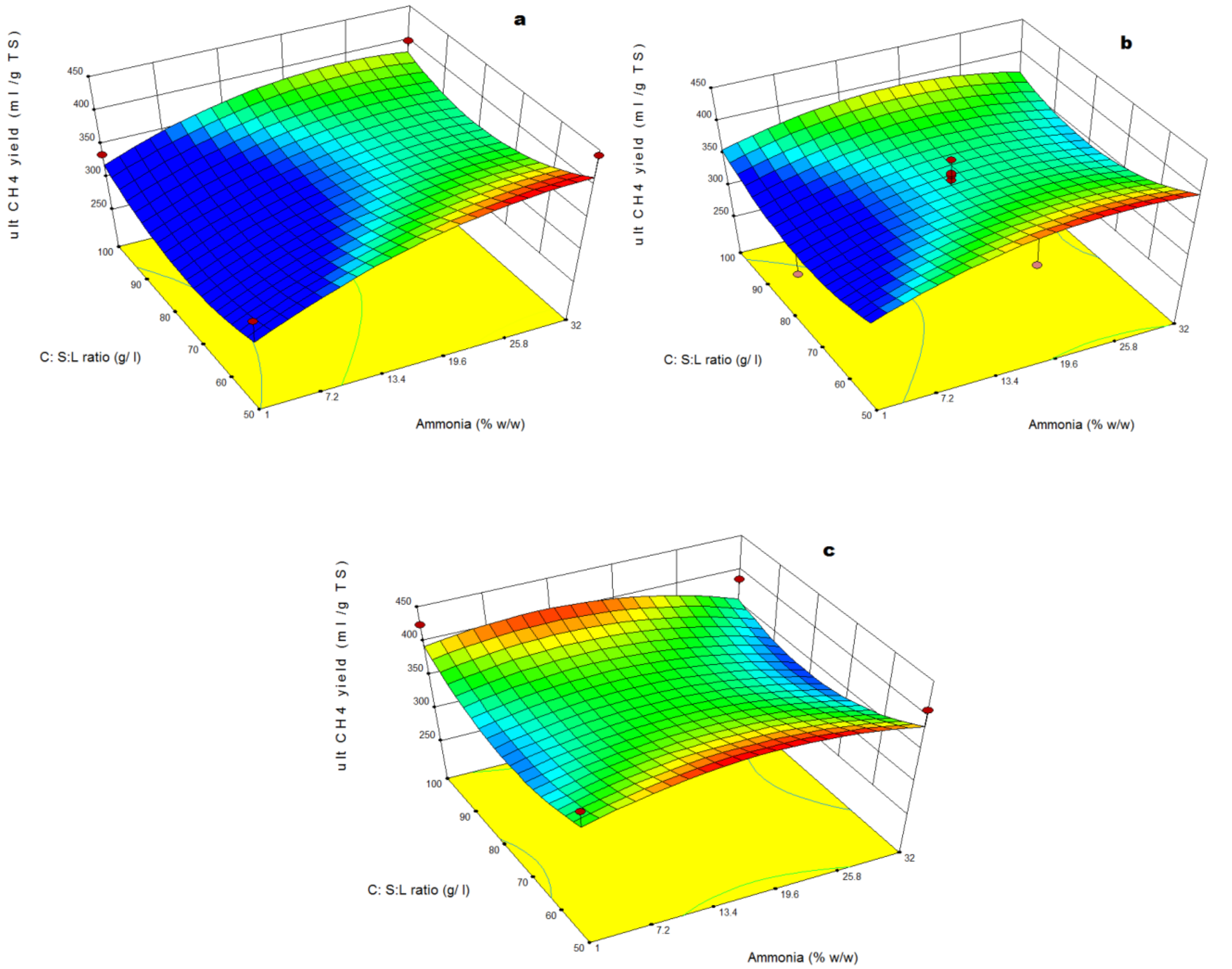

Figure 3. Response Surface graphs based on equation 4. The predicted ultimate CH_4 yield of AAS-treated wheat straw is plotted as a function of the NH_3 concentration and the S:L ratio. The duration of AAS is set constant in each graph, corresponding to 1, 4 and 7 days in a, b and c respectively. Dots in figures correspond to the experimental points.

The effect of the pretreatment on the ultimate CH_4 yield mainly shows how the final digestibility of the biomass is affected, while it can be a poor indicator for the increase of the CH_4 yield on a real AD process where the Hydraulic Retention Time (HRT) is usually limited or it is desirable to be limited to around 15-20 days [38]. Thus, the effect of the AAS parameters on the short term CH_4 yield is of interest from an application point of view. The short term CH_4 yields (17 days of digestion) of the BMP experiments of AAS-treated wheat straw varied from 223.40 ± 21.53 ml/g TS to 325.59 ± 6.96 ml /g TS, and the raw wheat straw resulted in an average of 228.25 ml/ g TS of CH_4 yield (Table 3). In all cases except experiment 13, the pretreatment resulted in an increase of the short term CH_4 yield, reaching a maximum of 51 % (experiment 4). Experiment 13 resulted in less short term CH_4 yield than the raw biomass (-7 %) as well as in a lower ultimate CH_4 yield. The main reason for this observation might be that practically AAS resulted in no net improvement of digestibility as the NH_3 concentration was very low (1 % w/w) and not compensated with the highest duration (4 days instead of 7), and the S:L ratio was set at the middle value (75 g/l). Nevertheless, the lower ultimate CH_4 yield indicates that some adverse effect might occur due to AAS.

Table 5 ANOVA table of model predicting the cumulative CH_4 yield obtained after 17 days of digestion (eq.4)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Block	1051.67	1	1051.67		
Model	11185.32	6	1864.22	16.25	< 0.0001
A-Ammonia	3471.05	1	3471.05	30.25	0.0001
D-Duration	2980.15	1	2980.15	25.97	0.0003
SL-S:L ratio	3.68	1	3.68	0.032	0.8608
A*D	1061.43	1	1061.43	9.25	0.0102
A^2	3262.17	1	3262.17	28.43	0.0002
SL^2	2202.31	1	2202.31	19.19	0.0009
Residual	1376.99	12	114.75		
Lack of Fit	903.89	8	112.99	0.96	0.5592
Pure Error	473.10	4	118.27		
Cor Total	13613.99	19			

Terms found to be statistically insignificant and excluded from the model: D·SL (p = 0.5196), A·SL (p =0.4418) D^2 (p = 0.8376)

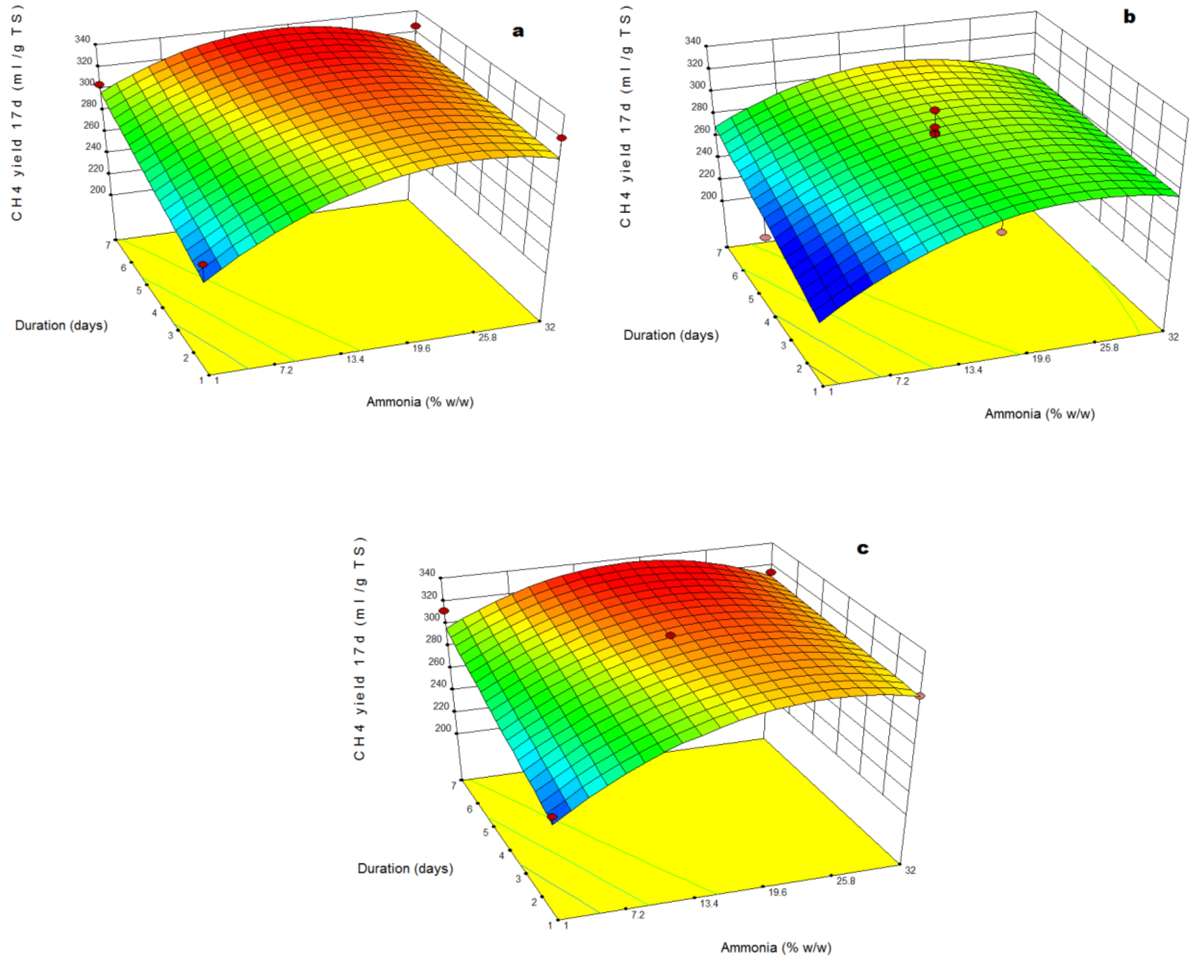

Figure 5 Response Surface graphs of equation 5. The surface represents the predicted CH_4 yield of AAS-treated wheat straw after 17 days of digestion as a function of the duration and NH_3 concentration under which the pretreatment takes place. The S:L ratio is set constant to 50 g/l, 75 g/l and 100 g/l in a, b and c respectively. Dots in figures correspond to the experimental points.

An empirical model was constructed for the short term CH_4 yield as a function of the AAS parameters (eq.5). The model was found to be highly significant ($p < 0.0001$) and no lack of fit was detected ($p = 0.5592$), (Table 5). The R^2 (0.89) was in good agreement with the adjusted R^2 (0.83) indicating that the terms included in the model are sufficient for describing the response. Thus, the model can be trusted for navigating the design space.

$$CH_4\ yield_{17} = 436.80 + 6.69 * A + 9.84 * D - 6.44 * SL - 0.25 * A * D - 0.14 * A^2 + 0.04 * SL^2 \text{ (eq.5)}$$

Where:

$CH_4 yield\ 17d$, the cumulative CH_4 yield of AAS-treated straw after 17 days of digestion, in ml/ g TS

A, the NH_3 concentration of the reagent, in % w/w in water

D, the duration of AAS, in days

SL, the solid-to-liquid ratio of the pretreatment mixture, in g wheat straw/ l reagent

The terms found to be significant on the short term CH_4 yield were the same as for the ultimate CH_4 yield, except for the interaction among the NH_3 concentration and the S:L ratio of AAS (A·SL), that appears not to be that influencing on the short term efficiency of the pretreatment. On the contrary, the NH_3 concentration (A) still remains the most influencing factor as well as the duration of AAS (D) and their interaction (A·D). The response surface graphs in Fig. 5 show how the prediction of the short term CH_4 yield is affected by the different levels of the two interacting parameters (A and D) at the three levels of S:L ratio. As it may be seen by comparing the three surface graphs (Fig. 5), the effect of the S:L ratio produces an elevation of the surface when it is set at 50 g/l or 100 g/l, and the middle S:L ratio (75 g/l) results to the lowest values for CH_4 yield at all combinations of NH_3 concentration and duration of AAS. While the lowest and highest S:L ratio appear to have a similar effect on the predicted CH_4 yield, the lowest S:L ratio has been observed to be the optimal according to the experimental results. Based on the graphs it appears that the optimal conditions of AAS for increasing the short term CH_4 yield of AAS-treated wheat straw correspond to 18 % w/w NH_3, 7 days of duration and 50 g/l. The prediction of eq. 5 at these conditions corresponds to 337.04 ± 11.12 ml/g TS.

3.4. Validation of empirical models and theoretical calculations

In order to validate the empirical models produced from the experimental data of this study (eq. 3, eq. 4, and eq. 5), the optimal conditions for maximizing the short term CH_4 yield were applied to wheat straw in three independent runs, and BMP experiments were set in triplicates for each run. The conditions applied were 18% w/w of NH_3 (aq.), 7 days of duration and 50 g wheat straw/ l reagent. The experimental results obtained by the wheat straw treated under these conditions resulted on average in 325.87 ± 16.74 ml/g TS short term CH_4 yield, 393.26 ± 17.42 ml/ g TS ultimate CH_4 yield and 20.36 ± 1.43 % COD solubilized. As shown in Table 6, the experimental results were all within the prediction range of each model. The hydrolysis rate of the wheat straw treated under optimal conditions was 0.1080 (R^2 = 0.9907), reaching nearly a 65 % increase in comparison to the raw wheat straw. The theoretical CH_4 yield of the wheat straw used in this study was found to be 415.17 ml/g TS (at STP conditions, 0°C, 1 atm), corresponding to 436.33 ml/ g VS (at STP). This value is similar to values reported in other studies, e.g. 444 ml/ g VS [39], 426 ml/ g VS [40], 432 ml/ g VS [13] and 436 ml/ g VS [41]. The digestion tests of raw wheat straw produced an average 212.68 ml/ g TS at STP conditions corresponding to 51.2% of the theoretical CH_4 yield, while the wheat straw treated under optimal AAS conditions resulted in 73.1% (303.63 ml/g TS at STP). In an earlier study where AAS was applied on wheat straw with 32% w/w for 3 days, a 41% increase of the short term CH_4 yield was reported [18]. AAS at low temperature under optimal conditions produced a 43% increase of the short term CH_4 yield as compared to the raw wheat straw. This confirms the strong interaction among NH_3 concentration and duration, as the increase of duration (from 3 to 7 days) permits reducing the concentration of NH_3 significantly (from 32 to 18% w/w) resulting to a slightly higher increase of the CH_4 yield.

In comparison to other pretreatments tested so far on wheat straw, AAS appears to have a relatively high influence on wheat straw. Nevertheless the highest increase of CH_4 yield reported so far from wheat straw has been by NaOH pretreatment [42], and corresponds to 111.6%. A recent review on pretreatments that have been tested on wheat straw and the resulted increase can be found in [4]. Alkaline reagents appear to be the most efficient on increasing the digestibility of lignocellulosic biomasses [43]. However, NaOH pretreatment would be associated to the additional cost of chemicals also for neutralizing the pretreatment mixture prior to digestion. Additionally, the resulting high sodium concentration might present a problem for applying the digestate to land [43]. When comparing the potential of a pretreatment it is important to take into account the costs associated with chemicals consumption. As commented in section 1, the high potential of AAS resides on the nature of this alkaline reagent that could permit an easier recycling. A techno-economic analysis is still pending though and should be carried out in order to estimate the feasibility of the suggested process (AAS at ambient temperature coupled to NH_3 recovery).

Table 6 Prediction of responses under optimal conditions of AAS and Validation of empirical models

Response	Model	Mean	Median	Observed	Std Dev	SE Mean	95% CI low	95% CI high
CH_4 yield$_{17}$	eq.5	337.04	337.04	325.87 ± 16.74	11.12	7.86	319.91	354.18
ultCH_4 yield	eq.4	408.61	408.61	393.26 ± 17.42	13.06	9.24	388.26	428.96
% sol COD	eq.3	21.42	21.42	20.36 ± 1.43	2.46	1.10	19.07	23.77

Std Dev stands for standard deviation as predicted by the model and CI for Confidence Interval. Observed values correspond to average values of triplicates ± standard deviation.

3.5. Effect of optimized AAS on composition of wheat straw

The effect of AAS on wheat straw pretreated under optimal conditions was further investigated by comparing its composition to the raw biomass (Table 7). A significant solubilization of the hemicellulose fraction was observed, indicated by a 60.8% and 67.5% reduction of the xylan and arabinan content of the solid matrix of wheat straw respectively. Part of the solubilized sugars were detected in the liquid fraction of the pretreated biomass together with a significant increase of the acetic acid content (1.52% TS from 0.33% TS from the raw biomass). The lignin fraction was also solubilized, resulting to a lignin removal of 9% from the solid matrix. Solubilization of hemicellulose and lignin might result to formation of inhibitory by-products, such as HMF, furfurals and lignin derivatives [25]. Nevertheless, these byproducts are usually formed by harsh pretreatment conditions where heat application or acids are involved [25,44]. Under mild conditions of AAS, it has been reported that no such by-products are formed [23,24]. Therefore, the fraction of hemicellulose not recovered in the form of oligosaccharides or free sugars in the liquid fraction probably resulted in other degradation compounds [44].

Table 7 Composition of raw wheat straw and optimally AAS-treated wheat straw

Component	Raw wheat straw (% TS)	AAS-treated wheat straw (% $TS_{initial}$)
Dry matter (% wet mass)	93.99 ± 0.14	3.70 ± 0.10
Glucan	41.07 ± 2.94	43.41 ± 1.27
Xylan	23.77 ± 1.18	9.32 ± 0.43
Arabinan	3.26 ± 0.10	1.06 ± 0.04
Total structural carbohydrates	68.10 ± 4.23	53.78 ± 1.66
Acid-insoluble lignin	16.24 ± 0.73	14.78 ± 0.93
Acid-soluble lignin	0.40 ± 0.00	2.17 ± 0.01
Total lignin	16.64 ± 0.73	16.95 ± 0.94
Extractives & volatiles	14.58 ± 0.04	20.31 ± 0.38
Water extractives	9.44 ± 0.23	17.58 ± 1.33
Ethanol extractives	2.13 ± 0.72	3.20 ± 0.37
Total Ash	5.32 ± 0.91	5.21 ± 0.01
Soluble sugars	0.69 ± 0.12	6.23 ± 0.04
Soluble Glucose	0.31 ± 0.03	0.78 ± 0.00
Soluble Xylose	0.55 ± 0.06	4.42 ± 0.02
Soluble Arabinose	0.16 ± 0.02	1.12 ± 0.00
Free sugars	0.33 ± 0.01	0.10 ± 0.02
Acetic Acid	0.33 ± 0.00	1.52 ± 0.01
NH_4^+-N content	0.01 ± 0.00	0.05 ± 0.00
Total Organic N	0.66 ± 0.03	1.02 ± 0.05
C/N *	63.21 ± 0.65	27.98 ± 3.89
Solid Recovery %**	-	97.96

*Unitless **Solid Recovery was calculated as g TS after pretreatment divided by the g TS before treatment and multiplied with 100.

An increased fraction of N was detected in the pretreated biomass, probably due to N chemical fixation that occurred during AAS. As commented in section 1, one of the benefits when using agricultural straws for AD is the high C/N ratio that permits improving the co-digestion with N-rich wastes. The decrease of the C/N ratio due to N chemical fixation during the pretreatment results thus to a reduced flexibility on co-digesting. Nevertheless, the C/N ratio of the pretreated biomass itself stands within the optimal conditions for AD [7]. Even though the extent of N chemical fixation observed might not present an inhibition problem for the AD process, the fate of the reagent-derived N should be further investigated.

Conclusions

Aqueous Ammonia Soaking (AAS) at ambient temperature was applied on wheat straw under different levels of NH_3 concentration, duration and S:L ratio in order to study the effect of these parameters on the CH_4 yield. A strong interaction among NH_3 concentration and treatment duration was observed that provides certain flexibility on a successful application of AAS on wheat straw. The conditions found to maximize the short term CH_4 yield (17 days) differed from the conditions that maximized the ultimate CH_4 yield. The optimal conditions for the short term CH_4 yield corresponded to 18% w/w NH_3 (aq.), 7 days of digestion and 50 g straw/l reagent, and led to a 43% increase as compared to the raw biomass. The significant solubilization of the solid matrix of wheat straw after pretreatment indicated the importance of using the whole fraction of the biomass (solid and liquid) for enhancing the CH_4 production. Compositional analysis of the optimally pretreated wheat straw showed that the fraction solubilized was mainly derived from hemicellulose, while a moderate lignin removal occurred.

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Paper V:

"Effect of optimized Aqueous Ammonia Soaking of manure fibers on continuous anaerobic digestion of swine manure"

“Effect of optimized Aqueous Ammonia Soaking of manure fibers on continuous anaerobic digestion of swine manure”

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Abstract

Swine manure mono-digestion often results into low biogas and methane productivity due to the low degradation rate of its solid fraction (manure fibers), and due to the high ammonia and water content. The Aqueous Ammonia Soaking (AAS) pretreatment of swine manure fibers has been proposed for overcoming these limitations. In this study, continuous anaerobic digestion (AD) of swine manure enriched with optimally AAS-treated manure fibers was compared to the AD of manure enriched with untreated manure fibers. AAS pretreatment of manure fibers under optimal conditions improved the biogas productivity and methane yield of manure enriched with pretreated fibers by 17% and 38% respectively. An improved reduction efficiency of all major organic components was observed, and the highest reduction corresponded to the cellulose fraction (60.3% as compared to 42.6%). Overall, the AAS pretreatment of manure fibers under optimal conditions was verified as a method for improving swine manure mono-digestion. Future investigation of the process proposed

at pilot scale should take place, in order to evaluate the process stability at higher organic loading rates than tested (1 g VS/l/d).

***Keywords**: manure, aqueous ammonia soaking, anaerobic digestion, pretreatment, CSTR*

1. Introduction

Swine manure is a major source of methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions, contributing significantly to the greenhouse effect. The global pig production sector is responsible for the emission of 668 million tons of CO_2-equivalents, and 27.4% of this is associated to manure management, mainly as CH_4 emissions during storage (Gerber et al., 2013). In presence of the available nutrients, the inherent microorganisms of manure degrade the organic matter remaining after the animal digestion, resulting to a series of degradation products, among them CH_4 and CO_2. The controlled microbial degradation of manure through the anaerobic digestion (AD) process is a commonly applied technology, during which the emissions are captured and can be used in the form of biogas as a renewable energy source. Additionally, the digested manure can be disposed in a safer manner as the C content has been stabilized to a significant extent. In Europe, around 65% of manure is handled in liquid form (slurry), containing a mixture of feces, urine, washing water and bedding material (Holm-Nielsen et al., 2009). As a result, the dry matter content of liquid manure is lower than 10%. This fact, in combination with the low degradation rate of manure and the high ammonia content, results in a poor biogas production and consequently in economically non-feasible AD processes (Asam et al., 2011).

In light of the forthcoming shortage of fossil fuels, a lot of focus has been given lately in manure-based AD processes mainly for boosting the biogas production. In turn, this has given rise to co-digestion practices (Weiland, 2010), where manure is enriched with easily degradable materials for improving the biogas production. Nevertheless, in countries like Denmark, the availability of these co-substrates is limited in comparison to the amounts of manure produced (Hamelin et al., 2011). Consequently, lower amounts of manure are treated anaerobically, increasing thus the negative environmental impacts from the disposal of untreated manure.

In order to facilitate the efficient manure management, a separation of manure to a solid (fiber) and liquid fraction is implemented in some countries (Foged et al., 2011). In Denmark the separation of manure is common, and ca. 90.000 tons of fibers are generated annually (Thygesen et al., 2014). Upon separation, the fiber fraction of manure that contains the accumulated recalcitrant organic content could be further pretreated for enhancing their bioconversion (Angelidaki and Ahring, 2000), while the liquid fraction, poor in organic matter and rich in nutrients such as N and K, can remain in farm to be used as a fertilizer. Subsequently, the pretreated fibers can be used to enrich raw manure for increasing the biogas production. This approach permits to increase the dry matter content of liquid manure (with fibers that present an improved digestibility) and to reduce the cost of manure transportation to biogas plants due to its reduced volume (Asam et al., 2011).

The pretreatment of manure fibers aims at overcoming the recalcitrance of their lignocellulosic structure that significantly slows down their digestion, facilitating thus a higher biogas and CH_4 production. A significant research effort is noted nowadays for identifying efficient pretreatments for improving the AD process of lignocellulosic biomasses. Among the pretreatments tested on swine manure fibers, Aqueous Ammonia Soaking (AAS), that has been tested on several biomasses for bioethanol (Kim et al., 2016; Kim and Lee, 2005) and for biogas production (Antonopoulou et al., 2015; Himmelsbach et al., 2010; Jurado et al., 2013a, 2013c; Mirtsou-Xanthopoulou et al., 2014; Song et al., 2014, 2012; Yu et al., 2014), presents certain characteristics that make it a promising pretreatment to be applied on manure fibers. Usually chemical pretreatments are not preferred as they result to be costly due to the consumption of chemicals. The main advantage of AAS, is the possibility of removing and recycling the only chemical used, NH_3, relatively easy due to its high volatility (Jurado et al., 2013c; Kim et al., 2016). In case a surplus of NH_3 is needed, this can be recovered from the digestate that presents an increased NH_3 concentration due to the mineralization of organic N during AD (Lymperatou et al., 2015). This process could be facilitated by using waste heat from Combined Heat and Power (CHP) plants that are often associated to biogas plants. Then again, given ambient temperature and pressure is applied during the pretreatment, low energy input is anticipated. Finally, a recent study showed that swine manure fibers respond greatly to this pretreatment and when applied under optimal conditions, a 244% increase of CH_4 yield can be obtained in batch AD (Lymperatou et al., paper III).

Batch experiments can be very useful on indicating the biodegradability rate of substrates as well as for a fast comparison of AD under different conditions or substrates. Nevertheless, as the majority of industrial scale digesters operate in continuous mode, continuous AD experiments can provide valuable information on the performance of a process that is closer to a real application (Carrère et al., 2016).

Following the efficiency of AAS on swine manure fibers demonstrated up to know from batch experiments, and due to the promising characteristics of this pretreatment, the present work aims at assessing the efficiency of continuous anaerobic digestion of manure enriched with optimally AAS-treated fibers, as compared with the efficiency of continuous anaerobic digestion of manure enriched with untreated fibers. Focus was given on the biogas and methane productivity of the digesters as well as on the reduction efficiency of the different organic fractions (carbohydrates, lipids, protein and lignin) of the enriched manure processes.

2. Materials & Methods

Feedstock

Swine manure and swine manure fibers were collected from Hashøj biogas plant (Sjæland, Denmark) and Limfjordens Bioenergi (Mors, Denmark) respectively. The manure fibers were separated in farm from raw swine manure by means of a mobile decanter centrifuge. All feedstocks were placed in closed containers and sealed bags and stored at -20 °C until used for the experiments. The main characteristics of the feedstocks used are shown in Table 1.

Aqueous Ammonia Soaking (AAS) Pretreatment

The AAS pretreatment of the swine manure fibers was performed at ambient temperature (20°C) and under optimal conditions (7% NH_3 w/w, 4 days, 0.16 kg/l) according to previous results and as described in Lymperatou et al. (paper III). After the end of the pretreatment, tap water of equal volume to reagent was added to the pretreated fibers and the NH_3 was removed by vacuum evaporation (130 mbar, 80 min).

The concentration of the remaining NH_4^+-N in the pretreated fibers was less than 0.5 g/l.

Experimental set up

Three CSTR-type digesters (3 l active volume) were used in the present study; all running under mesophilic conditions (37 °C). The first digester was fed only with swine manure serving as a reference process (Reference digester) for obtaining background data on the CH_4 productivity and yield of a process with similar characteristics fed only with manure. The inoculum for the digesters' start-up originated from the effluent of a lab-scale digester running on swine manure for 3 years under mesophilic conditions. After 120 days of operation of the Reference digester, the second (NP digester) and third digester (AAS digester) were inoculated simultaneously with a mixture of liquid from the Reference digester and accumulated effluent also from the Reference digester. The NP and AAS digesters were fed with a mixture of swine manure and manure fibers with a swine manure to fibers ratio of 2:1 (TS basis), where the fibers were non-pretreated and optimally AAS-treated respectively. The ratio used was chosen based on preliminary experiments, where a TS ratio of 1:1 produced operational problems due to clogging of the feeding tubing of the NP digester, so it was decided to reduce the fraction of fibers. The feed for the NP digester was diluted with tap water in order to achieve the same TS content and organic loading rate as the AAS digester. The experiments with the NP and AAS digesters lasted 125 days.

All three digesters were fed once per day by means of peristaltic pumps after rigorous mixing of the feed for 15 min. Feed mixtures were prepared twice per week and the feed tanks were kept at 4°C for reducing microbial degradation prior to digestion. Stirring of the digesters was intermittent and took place every 3 hours for 10 min. The heating of the digesters was achieved by water jackets with recirculating water from a thermostatic water bath.

Biogas production was measured with Ritter MilliGascounters (Ritter, Germany). The CH_4 content of the produced biogas, volatile fatty acids (VFAs), soluble COD, NH_4^+-N content, and pH were monitored weekly in all digesters. Samples for Total Solids (TS) and Volatile Solids (VS) determination were also collected weekly from the influent and effluent streams as well as from inside the digesters. The Total Suspended Solids (TSS) concentration inside the digesters and in the effluents was determined every two weeks. The solids retention time (SRT) was higher than the hydraulic retention time (HRT) in all digesters due to part of the effluent pipe that was vertical, permitting the re-settling of the solids when the digesters were not fed, (Jurado et al., 2016). The SRT was estimated based on the TSS measurements of samples taken from inside the digesters and from the effluents, as:

$$SRT = \frac{TSS_{reactor} * V_{reactor}}{Q_{out} * TSS_{effluent}} \text{ (eq.1)}$$

Where:

SRT the solids retention time in days

$TSS_{reactor}$ and $TSS_{effluent}$ are the concentrations of TSS in g/l inside the reactor and in the effluent respectively

$V_{reactor}$ is the active volume of the reactor in l

Q_{out} is the flow rate of the effluent in l/d

Analytical methods and compositional analysis

TS, VS, TSS, VSS and ash content were determined according to Standards Methods (APHA, 2005). Determination of soluble compounds was performed after centrifugation of samples at 10,000 rpm for 10 min and subsequent filtration of the supernatant through 0.45 μm. NH_4^+-N and soluble COD concentrations were quantified by LCK 305 and LCK 514 respectively. Free NH_3 content was calculated as (Hansen et al., 1998):

$$NH_3 - N = TAN * \left(1 + \frac{10^{-pH}}{10^{-\left(0{,}09018+\frac{2729.92}{273.15+T}\right)}}\right)^{-1} \quad \text{(eq.2)}$$

Where:

TAN is the total ammonia concentration ($NH_4^+ + NH_3$) in the digester in g/l

T is the temperature of the digester in °C.

Determination of CH_4 content in biogas was carried out by Gas Chromatography (GC82-22, Mikrolab Aarhus, Denmark). The GC was equipped with a Porapak Q packed column (6 ft. and I.D. 3 mm), coupled with a Thermal Conductivity Detector (TCD) and N_2 was used as a carrier gas. All gas volumes reported correspond to STP conditions (0°C, 1 atm).

Compositional analyses of manure and digester effluents were performed on samples taken during the period of 70-80 days of operation of the digesters. The procedure followed for quantification of structural carbohydrates, acid-insoluble lignin, water and ethanol extractives, and soluble sugars of the samples is described in Lymperatou et al. (paper III). The composition of the influents of the enriched manure digesters was calculated based on the composition of the manure fibers and the composition of raw manure used and taking into account the TS ratio of manure and manure fibers. Values reported for cellulose and hemicellulose correspond to the sum of glucose and sum of xylose and arabinose respectively. Samples for VFA analysis were acidified with H_2SO_4 (10 % w/w), centrifuged at 10,000 rpm for 10 min and filtered through 0.20 μm. Determination of sugars and VFAs was performed by High Performance Liquid Chromatography (HPLC) equipped with a refractive index and an Aminex HPX-87H column (BioRad) at 63 °C. A solution of 12 mM H_2SO_4 was used as an eluent at a flow rate of 0.6 ml/min. Total N content was determined through elemental analysis (EA3000, EuroVector, Italy) with acetanilide used as a standard. The proteins content was determined by subtracting the TAN content from the total N content, and multiplying by the factor 6.25 (Galí et al., 2009). The lipids were determined as the mass of extractives after 24 hours of extraction with ethanol 96 % v/v using a Soxhlet apparatus ((EV6 ALL/16 No. 10-0012, Gerhardt, Germany).

Calculations and Assumptions

The reduction efficiencies of the major biomass components (cellulose, hemicellulose, proteins, lipids and lignin) were calculated as:

$$\%\,reduction = \frac{x_{i,in} - x_{i,out}}{x_{in}} * 100 \text{ (eq.3)}$$

Where:

$x_{i,in}$ the concentration of each component i in g/kg in the influent

$x_{i,out}$ the concentration of each component i in g/kg in the effluent

The theoretical CH_4 yield was calculated based on the composition and according to Buswell's formula (Symons and Buswell, 1933):

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4 \text{ (eq.2)}$$

The compositions for each component were assumed to be: $C_6H_{10}O_5$ for cellulose, $C_5H_{10}O_5$ for hemicellulose, $C_5H_7O_2N_1$ for proteins, $C_{57}H_{104}O_6$ for lipids, and $C_2H_4O_2$ for VFAs, as suggested by Møller et al. (Møller et al., 2004).

3. Results & Discussion

Process characteristics and stability

The HRT of the digesters was aimed at 17 days, which is similar to typical HRTs applied at Danish manure-based biogas plants (Mladenovska et al., 2006). Nevertheless, due to the inhomogeneity of the feed, the volume pumped daily into the digesters varied. This effect was more evident in the NP and AAS digesters due to larger fiber fractions. Additionally, the accumulation of solids in the digesters due to construction limitations (see section 2.2) resulted in significantly different SRTs between the reference digester and the mixture-based digesters (Table 2). Especially in the case of the NP digester, a sharp increase of the TS content inside the digester was observed after 95 days of digestion. Thus, the average performance of the NP and AAS digesters after 65 days (when steady state was assumed) and up to 95 days is presented (Table 2). During this period the SRT of the NP and AAS digesters was similar, therefore their performance could be compared more properly.

Table 1 Composition of manure and non-pretreated and AAS treated fibers used in this study

Component	Swine manure	Non-pretreated manure fibers[b]	AAS-treated manure fibers[b]
TS (% wet mass)	2.2	3.2[a]	3.1
VS (% wet mass)	1.5	2.2	2.2
Cellulose (% TS)	12.3	30.4	31.2
Hemicellulose (% TS)	9.2	21.7	16.1
Proteins (% TS)	22.9	15.1	19.9
Lipids (% TS)	7.7	7.8	5.9
Lignin (% TS)	15.8	16.6	16.7
TAN (% TS)	1.09	0.37	0.99

[a] after dilution with tap water [b] adapted from Lymperatou et al. (paper III).

In general, the enrichment of swine manure with manure fibers (both non-pretreated and AAS-treated) resulted to a slight increase of organic matter in the mixtures. The highest increase of the organic fractions in the influents corresponded to carbohydrates (cellulose and hemicellulose fractions), as fibers were characterized by significantly higher carbohydrates concentration than manure (Table 1). Regarding the differences between the influents of the enriched digesters, the NP digester presented higher hemicellulose content and a less apparent protein content than the AAS digester. This is because during AAS, part of the reagent N was bound to organic matter, resulting to a higher organic N detected in the pretreated biomass in comparison to the untreated, while the hemicellulose fraction was partly solubilized and part of it was converted to degradation products not detected as sugars (Lymperatou et al., paper III).

Swine manure often contains high TAN concentrations, which in combination to the mineralization of organic N during AD, increases the risk of inhibition of the methanogenesis step (Hansen et al., 1998). Therefore, mono-digestion of this feedstock is often discouraged (Sawatdeenarunat et al., 2015). The TAN concentration in the reference digester, which was fed only with swine manure, was 2.73 g/l and the free NH_3 concentration which is considered to be the main inhibitor (McCarty and McKinney, 1961; Yenigün and Demirel, 2013) was 0.45 g/l. The addition of fibers, both in the NP and AAS digester, led to a reduction of the TAN concentration (Table 2). This was expected, as besides the lower TAN concentration of the fibers, the mixture-based digesters had lower organic N content as shown by the C/N_{org} ratios (11.08 and 10.24 for the NP and AAS digester respectively in comparison to 9.44 for the Reference digester), (Table 2). Generally the threshold value reported in literature at which NH_3 inhibition begins, varies significantly and is dependent mainly on the pH, the temperature, the TAN concentration and the acclimation of the inoculum (Chen et al., 2014). According to a recent review by Yenigün and Demirel (2013), a TAN concentration above 1.7-1.8 g /l is inhibitory under mesophilic conditions without acclimation of the inoculum. The NP digester was the only digester with such low concentration. However, none of the processes should be restricted by the TAN concentration, as the initial inoculum originated from a digester running for 3 years on swine manure with TAN concentrations similar to the Reference digester's concentration used and thus, could be considered well acclimated (Fotidis et al., 2014). The concentration of VFAs, which is considered to be an indicator for evaluating the stability of the process and shows whether inhibition occurs, was relatively low during the entire period of experiments in all processes in this study. The AAS digester was characterized by a slightly higher average VFA concentration (0.26 g/l) in comparison to the Reference digester fed only with manure (0.22 g/l), which could be explained by the addition of organic matter which could be degraded faster due to the AAS pretreatment. On the contrary, the NP digester presented the lowest VFA concentration (0.19 g/l).

In a previous study, where manure fibers were added to pig manure, a significant increase in the TAN concentration was observed, producing inhibition of the process when increasing the substitution of manure with fibers up to 60% (Møller et al., 2007b). This difference was mainly attributed to the higher total N concentrations of manure fibers in comparison to raw manure (Møller et al., 2007b). In this study, both the organic N (proteins) and TAN content of manure fibers was lower than that of raw manure (Table 1), and in contrast to the thermophilic temperature and high organic loading rate (OLR) applied in the previous study (Møller et al., 2007b) the processes took place under mesophilic conditions where the process is less sensible to NH_3 inhibition (Angelidaki and Ahring, 1994) and with a low OLR.

The efficiency of separating the organic N content of manure depends on the technology used and, generally, it is increased with an increased efficiency of solids separation (Møller et al., 2007a, 2002). On the other hand, the TAN concentration remains to the liquid fraction regardless the separator used (Møller et al., 2002). Thus, if no further pretreatment of fibers with high N concentration is followed, their use for enriching untreated manure might result to be prohibitive. The separation and AAS pretreatment of fibers for the enriching of raw manure can provide certain flexibility on the final total N content of the influent, given that an NH_3 removal step is necessarily applied after AAS. The source of manure fibers and the efficiency of solids separation could determine whether the organic N in the fibers is lower than in manure, while the NH_3 removal step following an AAS pretreatment can permit a better control of the initial TAN concentration of the feed. In this study, the NH_3 removal was not optimized. Nevertheless, in a future application of AAS, the latter should be optimized by taking into account also the economics of the process, for evaluating the feasibility of further reducing the TAN concentration of manure fibers.

Biogas and Methane production and yield

The AAS digester generally presented an improved biogas production compared to the rest of the digesters as shown in Table 2. The average biogas productivity of the AAS digester was 0.48 l/l/d, corresponding to a 17% increase as compared to the productivity of the NP digester (0.41 l/l/d). In comparison to the reference digester (0.43 l/l/d), substitution of 1/3 of the feed TS with AAS fibers resulted in a 12% increase of productivity. In a previous application where manure fibers treated with AAS under different conditions (32% w/w NH_3 for 3 days) were incorporated to swine manure and digested in continuous AD (Jurado et al., 2016), a 22% increase of biogas productivity was observed in comparison to a digester fed only with swine manure. Nevertheless, this increase resulted from the comparison of two digesters with different HRTs. Additionally, the TS ratio of manure to fibers fed to the digester was lower (0.52/0.48) than in this study (2/1), and thus a direct comparison of the results would be misleading. From a point of view of balanced nutrients, a higher fraction of fibers than manure is preferable as this would increase the C/N ratio. However, due to the limitations of the set up in this study (see section 2.2), this was not possible. Interestingly, the NP digester presented lower biogas productivity than the Reference digester, indicating that reducing the TAN concentration was not sufficient for improving the biogas efficiency of the process. This shows that the degradability of manure was a more important factor limiting the biogas production than the TAN concentration.

The CH_4 productivity of the digesters presented similar trends, with the AAS digester performing better than the rest of the digesters. Nevertheless, the addition of fibers (both untreated and AAS-treated) resulted in a reduction of the content of biogas in CH_4 as shown by the smaller differences in CH_4 productivity as compared to the biogas productivity (Table 2). This was probably a result of the higher fraction of carbohydrates in the mixture-based digesters, that stoichiometrically produce a lower CH_4/CO_2 ratio in comparison to lipids and proteins in which manure was richer (Table 1). Still, the highest CH_4 yield per g TS_{fed} was observed in the AAS digester. In Fig 1 the CH_4 yields of the three digesters are shown during the period of 65-125 days of operation. The high standard deviation observed in the graph is a consequence of the variable daily feed volume due to inhomogeneity, as commented in Section 3.1.

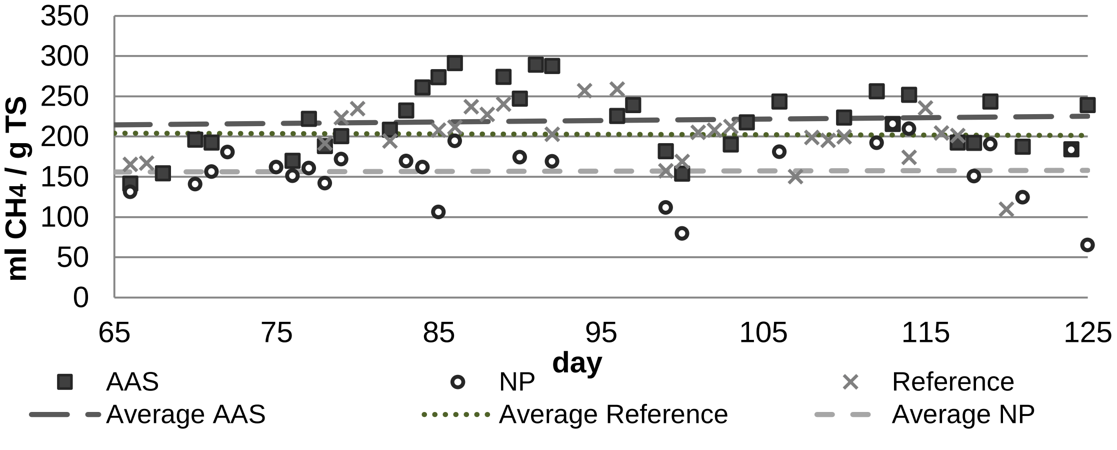

Figure 1 Methane yield of digesters NP (fed with manure and non-pretreated fibers), AAS (fed with manure and AAS-treated fibers) and Reference digester (fed only with manure)

Table 2 Characteristics of digesters during the period of 65-95 days of operation

Characteristic	NP digester	AAS digester	Reference digester
Feed ratio g TS manure: g TS fibers	2:1	2:1	1:0
C/N_{org} of influent	11.1	10.2	9.4
Organic Loading Rate (g VS/l/d)	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.1
Hydraulic Retention Time (d)	18.2 ± 1.1	17.9 ± 1.3	17.6 ± 0.1
Solid Retention Time (d)	26.7 ± 1.1	25.9 ± 1.9	20.5 ± 0.3
VFA concentration [a] (g/l)	0.19 ± 0.07	0.26 ± 0.07	0.22 ± 0.01
pH [a]	8.1 ± 0.0	8.1 ± 0.1	8.2 ± 0.3
Soluble COD [a] (g/l)	2.24 ± 0.07	2.34 ± 0.23	3.08 ± 1.34
TAN concentration [a] (g/l)	1.82 ± 0.26	2.04 ± 0.01	2.73 ± 0.05
Free NH_3 [a] (g/l)	0.25 ± 0.03	0.29 ± 0.01	0.46 ± 0.01
TS [a] (g/l) inside the digester	26.63 ± 0.95	22.17 ± 1.53	26.61 ± 1.00
VS [a] (g/l) inside the digester	16.40 ± 0.69	13.24 ± 1.01	15.29 ± 0.83
TSS [a] (g/l) inside the digester	20.35 ± 0.07	17.05 ± 0.07	16.70 ± 0.64
Biogas productivity (l/l/d)	0.41 ± 0.08	0.48± 0.06	0.43 ± 0.06
Methane productivity (l/l/d)	0.25 ± 0.05	0.30 ± 0.04	0.28 ± 0.04
Methane Yield (ml/g TS_{fed})	156 ± 37	215 ± 40	204 ± 34
Methane Yield (ml/ g VS_{fed})	222 ± 54	314 ± 61	330 ± 61

[a] Data correspond to average values from samples taken from inside the digesters accompanied by the standard deviation.

The theoretical maximum yields of the mixture-based digesters were calculated to be 377 ml/g TS_{fed} (538 ml/g VS_{fed}) and 365 ml/g TS_{fed} (533 ml/g VS_{fed}) for the NP and AAS digester respectively, while the experimentally obtained were 156 ml CH_4/g TS_{fed} and 215 ml CH_4/g TS_{fed} respectively. These correspond to a 41% and 59% of the theoretical yields of the NP and AAS digesters respectively, and to a 37.8% increase of the CH_4 yield in the AAS digester compared to the NP digester. Interestingly, the highest CH_4 yield per gVS_{fed} corresponded to the Reference digester. This was a result of the lower VS/TS ratio of manure in comparison to both NP and pretreated fibers, and indicates that the manure fibers were more recalcitrant (less hydrolysable) than the swine manure used in this study.

Assuming that the CH_4 yield of swine manure in the enriched digesters was the same as in the Reference digester and given that the feed ratio was (2 g TS manure)/ (1 g TS fibers), it was calculated that 59.7 ml/g TS_{fed} and 235.7 ml/g TS_{fed} corresponded to the NP and AAS-treated manure fibers respectively. Following this assumption, a 295% increase was obtained from AAS at continuous mode, which is higher than the increase found in batch experiments (Lymperatou et al., paper III). However, this estimation is indicative, as small variations of the CH_4 yield of manure affect significantly the calculated values of the CH_4 yields of manure fibers. Additionally, it has to be highlighted that the actual yields do not correspond to 18 days of digestion in the continuous processes, as the SRTs were ca. 26 days (Table 2). In batch experiments, it was observed that the AAS-treated fibers produced the majority of CH_4 in the first 2 weeks, while the NP fibers required more than 40 days of digestion (Lymperatou et al., paper III). Thus, if no retention of solids occurred, the difference of the CH_4 yield of the untreated and pretreated fibers could be expected to be higher.

Reduction efficiency of major organic components

The composition of the effluents of the NP and AAS digesters was analyzed in order to better estimate the effects produced by the optimized AAS pretreatment. Figure 2 shows the composition of the influents and effluents in the major organic components expressed as a percentage of VS. The organic matter of the effluents from both digesters (NP and AAS) appears to have been affected mainly in the carbohydrate content, while the fractions of the rest of components (lipids, proteins and lignin) were similar to the fractions of the organic matter of the influents (Figure 2). The AAS effluent clearly contained a reduced content of carbohydrates expressed in % VS, while the lignin content is similar to the influent fraction. As a result the cellulose/lignin ratio, which has been proposed to be one of the maturity indicators for organic substrates (Nkoa, 2014), changed from 0.98 to 0.75. On the contrary, the reduction of the cellulose/lignin ratio from the NP digester was considerably lower (0.97 in the influent to 0.90 in the effluent).

The efficiency of VS reduction of the NP and AAS digesters along with the reduction efficiencies of the major organic components are shown in Table 3. It is important to mention here that the VS increase due to the growth of microbial biomass has not been taken into account for the calculations. However, as the aim of this study was to compare the reduction efficiencies of the different organic components in the two digesters (NP and AAS), this was considered not to affect significantly the evaluation.

The AAS digester presented a higher efficiency on reducing the organic matter of the feed (50.7% reduction of VS) than the NP digester (45.8%). Among the two processes, the AAS digester presented higher reduction efficiencies in all major organic components of the feed. The highest difference was observed in the carbohydrate fraction where the digestion of manure enriched with pretreated fibers resulted in a 60.3% and 65.3% reduction of the cellulose and hemicellulose fractions respectively, in comparison to 42.6% and 54.1% from the digestion of manure with NP fibers. This corresponds to an increase of reduction efficiency of 42% and 21% for cellulose and hemicellulose respectively. This was expected as AAS affects mostly the lignocellulosic fraction of the biomass, by increasing the efficiency of polysaccharides hydrolysis. As reported in earlier studies, the main mechanism of AAS on swine manure fibers appears to be a swelling effect (Jurado et al., 2013b) together with a significant solubilization of the hemicellulose fraction (Lymperatou et al., paper III). This results to both the cellulose and hemicellulose fractions being more accessible for microbial degradation.

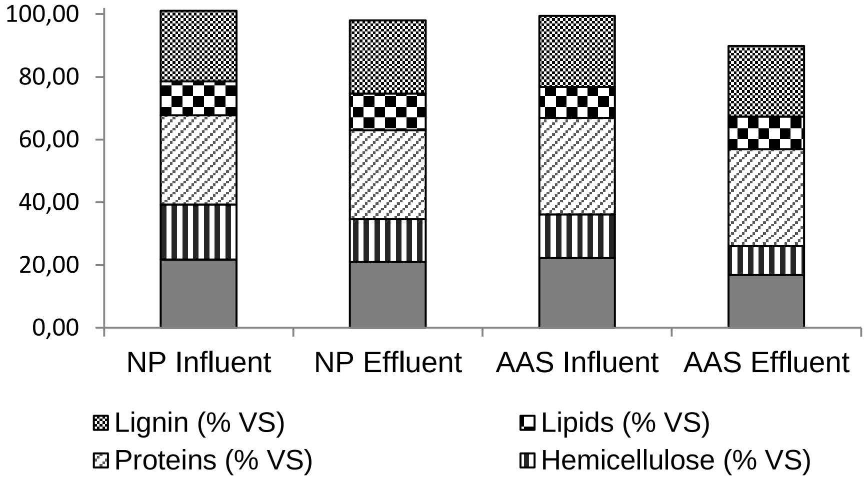

Figure 2 Composition of influents and effluents of NP and AAS digesters expressed in % of VS.

Besides the improved carbohydrate removal, AAS appears to have facilitated the reduction of lipids and proteins as well. A slight solubilization of organic N (associated to proteins) (Lymperatou et al. paper III) and decrease of ethanol extractives (associated to lipids) (Table 1) were observed after AAS, probably facilitating their further degradation. The reduction of lignin was also significantly affected by the AAS treatment, as it reached 48.2% in the AAS digester in comparison to 38.5% in the NP digester. It is known that lignin is not significantly removed from manure fibers after optimal AAS (Lymperatou et al., paper III), thus this could be a result of the swelling of the fibers that facilitated microbial access during AD, resulting in degradation products. Generally, lignin is considered to be recalcitrant to bioconversion and negatively correlated to CH_4 production (Monlau et al., 2012; Triolo et al., 2011). Nevertheless, upon degradation certain byproducts have been reported to be converted into CH_4 under anaerobic conditions (Barakat et al., 2012).

Table 3 Concentration of major organic components of Influents and Effluents of digesters and reduction efficiency of AD (data correspond to samples taken during 70-80 days digestion)

Component	NP digester			AAS digester		
	Influent (g/kg)	Effluent (g/kg)	% reduction	Influent (g/kg)	Effluent (g/kg)	% reduction
VS	21.87 ± 0.53	11.85 ± 1.93	45.8	20.32 ± 1.96	10.02 ± 2.16	50.7
Cellulose	4.76 ± 0.00[a]	2.74 ± 0.02	42.6	4.53 ± 0.11[a]	1.80 ± 0.11	60.3
Hemicellulose	3.83 ± 0.01[a]	1.76 ± 0.02	54.1	2.81 ± 0.01 [a]	0.98 ± 0.06	65.3
Proteins	6.21 ± 0.79[a]	3.70 ± 0.03	40.5	6.25 ± 1.20[a]	3.27 ± 0.03	47.7
Lipids	2.37 ± 0.18[a]	1.52 ± 0.13	35.6	2.01 ± 0.01[a]	1. 11 ± 0.19	44.6
Lignin	4.92 ± 0.36[a]	3.03 ± 0.00	38.5	4.61 ± 0.35[a]	2.39 ± 0.10	48.2
VFAs	6.52 ± 0.54	0.19 ± 0.07	97.1	6.20 ± 0.42	0.26 ± 0.07	95.8

[a] Estimated through mass balance

Overall, AAS appears to have affected positively the removal of organic components of manure fibers under mesophilic AD. The increased biogas production in the AAS digester also indicates that a larger part of manure fibers was digested as a result of the pretreatment. However, partial solubilization of the hemicellulose fraction was observed after the AAS pretreatment, which was not recovered in the form of sugars (Lymperatou et al. paper III). Thus, degradation byproducts that were not detected by the methods employed might have been produced, and their contribution to CH_4 was not possible to assess. Future research should include a detailed analysis for degradation products in order to assess their fate during AD.

4. Conclusions

Aqueous Ammonia Soaking (AAS) under optimal conditions allowed for a significantly improved conversion of the organic matter of swine manure fibers during manure-based anaerobic digestion (AD). The reduction efficiency of all major organic components was improved with the cellulose reduction reaching 60.1% compared to 42.6% for the untreated fibers. By substituting 1/3 of manure with optimally AAS-treated fibers, 18% and 37.6% increase of biogas productivity and methane yield were obtained as compared to substitution with non-pretreated fibers. Additionally, no stability issues were observed and the swine manure enriched with separated manure fibers (both untreated and AAS-treated) reduced significantly the risk of NH_3 inhibition during mesophilic anaerobic digestion.

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